The effect of biological and anthropogenic sound playbacks and self-generated movement on the hearing sensitivity of the Oyster Toadfish, *Opsanus tau*

A THESIS
SUBMITTED TO THE FACULTY OF THE
UNIVERSITY OF MINNESOTA
BY

Loranzie Samuel Rogers

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Allen F. Mensinger, Ph.D.

August 2019

Acknowledgments

I would like to acknowledge my advisor and mentor, Dr. Allen F. Mensinger. The unwavering support and guidance that Dr. Mensinger has provided throughout my time in his research lab has had a remarkable impact on my scientific development. I am incredibly thankful for the countless opportunities that Dr. Mensinger has provided.

I would like to thank my co-authors, Dr. Rosalyn L. Putland and Jacey C. VanWert, for their substantial help with these research projects and manuscript revisions. I would also like to acknowledge my committee members, Dr. Thomas Hrabik, Dr. Jennifer Liang and Dr. John Pastor for their support during the duration of my master's degree. Additionally, I would like to thank all of the current and previous members of the Mensinger Lab for their support throughout the years.

Lastly, I would like to thank the Biology Department at the University of Minnesota Duluth, the Diversity of Value and Excellence fellowship through the University of Minnesota and the National Science Foundation Graduate Research fellowship for financial support.

Abstract

Within the fish inner ear, three otolithic end organs (utricle, saccule and lagena) serve both auditory and vestibular roles. The Oyster toadfish (*Opsanus tau*) is a vocal fish species that has been extensively studied to understand both the vestibular and auditory functions of the fish inner ear. Previous studies, however, have primarily been conducted on restrained or stationary fishes so it remains unclear how self-generated movement impacts the otolithic end organs. Additionally, the effects of self-generated and anthropogenic sound on fish hearing remains to be known. To address these questions, microwire electrodes were inserted into utricle of free-swimming toadfish using an implantable micromanipulator. Experiments measured the neural response of the utricle to playbacks of conspecific vocalizations at variable speeds while swimming. During movement, fish remained sensitive to conspecific playbacks, indicating that the inner ear can detect auditory stimuli during movement. Additionally, acoustic evoked potential recordings were conducted to measure the auditory sensitivity of toadfish pre- and post-exposure to conspecific vocalizations and anthropogenic sound. Toadfish exhibited auditory sensitivity between 100 and 500 Hz, which overlaps the frequency range of conspecific vocalizations and anthropogenic sound, such as those generated by ship traffic. Exposure to conspecific vocalizations had no significant effect on toadfish auditory sensitivity; however, exposure to anthropogenic sound caused significant auditory impairment that was sustained for at least 3 days. For vocal fishes, the ability to detect and localize conspecific vocalizations is critical for their reproductive success. In the following chapters, I show that toadfish are capable of sound detection while

swimming and after exposure to conspecific vocalizations, but their hearing is impaired by anthropogenic sound.

Table of Contents

Acknowledgements i

Abstract ii

List of Tables v

List of Figures vi

Foreword viii

Chapter 1: Introduction 1

Chapter 2: The effect of biological and anthropogenic sound on the auditory sensitivity of Oyster Toadfish, *Opsanus tau* 11

Chapter 3: The effects of multimodal input on utricular sensitivity in free-swimming toadfish, *Opsanus tau* 39

Chapter 4: Future Investigations for Fish Sound Detection 70

References 73

List of Tables

Chapter 2:

Table 1: Particle acceleration level (dB re. 1 ms^{-2}) threshold shifts during serial testing after exposure to 1 hr anthropogenic sound 36

Table 2: Sound pressure level (dB re. 1 μPa) threshold shifts during serial testing after exposure to 12 hr anthropogenic sound 37

Table 3: Particle acceleration level (dB re. 1 ms^{-2}) threshold shifts during serial testing after exposure to 12 hr anthropogenic sound 38

List of Figures

Chapter 2:

Figure 1: Sound exposure 27

Figure 2: Particle acceleration levels (dB re. 1 ms^{-2}) across all frequencies used during AEP testing 29

Figure 3: Exposure tank particle acceleration (dB re. 1 ms^{-2}) and sound pressure level (dB re. 1 μPa) sound maps 30

Figure 4: Representative auditory evoked potential (AEP) responses 31

Figure 5: Minimum baseline levels needed to evoke an AEP response 32

Figure 6: Minimum baseline levels needed to evoke an AEP response following 1 hr. anthropogenic sound exposure 33

Figure 7: Minimum baseline levels needed to evoke an AEP response following 12 hr. anthropogenic sound exposure 34

Figure 8: Minimum baseline levels needed to evoke an AEP response following 12 hrs. of boatwhistle playbacks 35

Chapter 3:

Figure 1: Schematic of experimental set-up 59

Figure 2: Spontaneous activity of afferent utricular fibers 60

Figure 3: Utricular response to pure tone stimuli 61

Figure 4: Utricular response of stationary fish to boatwhistle playbacks 62

Figure 5: Utricular firing rates plotted against sled velocity and acceleration 63

Figure 6: Mean firing rates of utricular afferents during sled movements 64

Figure 7: Representative utricular afferent recordings during sled movement ... 65

Figure 8: Utricular afferent phase histograms in response to boatwhistle playbacks during sled movement 66

Figure 9: Particle acceleration level (dB re. 1 ms^{-2}) measurements 67

Figure 10: Utricular afferent firing rates during forward swimming 68

Figure 11: Free-swimming utricular afferent activities during boatwhistle playbacks 69

Foreword

This thesis is submitted as partial fulfillment for the degree of Master of Science in the Integrated Biosciences Graduate Program at the University of Minnesota Duluth. Manuscripts based on Chapters 2 and 3 are currently submitted for publication. Co-authors for Chapter 2 are: Dr. Rosalyn L. Putland and Dr. Allen F. Mensinger. Co-authors for Chapter 3 are: Jacey C. VanWert and Dr. Allen F. Mensinger.

Chapter 1: Introduction

The underwater environment is filled with numerous biotic (i.e. vocalizing organisms) and abiotic (i.e. wind, rain, water currents) factors that might affect the survival and reproductive success of aquatic organisms (Slabbekoorn et al., 2010). To facilitate the detection of critical ecological signals, aquatic organisms rely on an array of sensory systems to integrate and appropriately respond. The reliance on any individual sensory system depends heavily upon the environmental conditions (i.e. light levels and water flow rates) a given aquatic organism inhabits (Niven and Laughlin, 2008). However, one of the most evolutionarily conserved sensory systems in vertebrate aquatic organisms is the auditory system (Fay and Popper, 2000).

Among vocal fishes, the auditory system facilitates acoustic discrimination (Jacobs and Tavolga, 1968; Winn, 1972; Ladich and Fay, 2013), sound source localization (Zeddies et al., 2010, 2012) and the detection of their soundscape (Popper et al., 2003a; Fay, 2008). This array of functions is mediated via sensory hair cells that are located within the inner ear and the mechanosensory lateral line; however, the contribution of each system in mediating these functions is still unclear. Thus, a goal for aquatic neuroethologists has been to characterize the functions of both the lateral line and inner ear systems while fishes are normally behaving (Russell and Roberts, 1974; Tricas and Highstein, 1991; Maruska and Tricas, 2004; Zeddies et al., 2012; Chagnaud et al., 2017; Mensinger et al., 2019).

More recently, aquatic neuroethologists have become interested in how changes within the aquatic environment may impede auditory functions and fish's normal behaviors (Popper et al., 2003b; Radford et al., 2014). This research has suggested the

question: how do aquatic organisms retain auditory sensitivity following prolonged exposure to biological sounds, such as conspecific vocalizations or anthropogenic sound?

Underwater sound

Underwater sound, much like in air, travels as a longitudinal wave through the water medium. Underwater sound propagates faster and attenuates more slowly than sound in air due to the high molecular density of water (Urick, 1967). Underwater sound is defined as both sound pressure and particle motion. Sound pressure is the fluctuation of pressure deviations localized within regions of the longitudinal wave and is a scalar measurement of sound that travels omnidirectionally. Particle motion is the vibration of particles within the medium parallel to that of sound propagation and is a vector measurement of sound that carries directional information about the sound source (Larsen and Radford, 2018).

The sounds that make up a given acoustic environment have been termed soundscapes (Southworth, 1969). Soundscapes have been defined into three taxonomic groups (biophony, geophony and anthrophony), and are the main areas of focus for the field of soundscape ecology (Pijanowski et al., 2011). Biophony is characterized as both passive and active sounds produced by organisms, while geophony is composed of all nonbiological ambient sounds (i.e. wind, rain and breaking waves) and anthrophony is any sounds created by humans (i.e. aquatic vessels, on/off shore construction and seismic airguns) (Krause, 1987; Pijanowski et al., 2011).

Fish hearing

The ability to detect sound is a prerequisite for acoustic communication; however, not all fish detect the same sound characteristics. It is well known that all fish detect

particle motion components of sound, however, only approximately one third of fishes are able to detect the pressure component of sound (Slabbekoorn et al., 2010). Thus, fishes have been categorized on a continuum of pressure detection mechanisms ranging from fishes that are only sensitive to particle motion (i.e. flatfish) to fishes that extensively utilize pressure detection (i.e. otophysan fishes), with several intermediate groups between (Popper and Fay, 2011; Putland et al., 2018b). The overall detection of underwater sounds is mediated via the displacement of ciliary hair bundles in two sensory systems; the mechanosensory lateral line and the inner ear auditory systems (Harris and van Bergeijk, 1962; Popper and Fay, 1973).

Hair cells

Hair cells are the functional sensory cell of the inner ear and lateral line, and are composed ciliary bundles that consist of numerous small stereocilia and a single elongated kinocilium (Flock, 1965; Hudspeth, 1985). Hair cells function via the displacement of ciliary bundles and are innervated by afferent (excitatory) fibers and efferent (inhibitory) fibers. Displacement of these hair bundles depolarizes the cellular membrane resulting in the generation of action potentials in these fibers (Katz, 1969). The resulting action potentials will propagate along the nerve and will be encoded by the central nervous system (Purves et al., 2001). While action potentials are generated by both the lateral line and inner ear systems and processed by the central nervous system, there is some variation in the degree of sensory system sensitivity. The lateral line is most sensitive to low-frequency sounds up to distances of one or two body lengths, while the inner ear is sensitive to a wider range of frequencies originating at much greater distances (Popper and Fay, 2011).

Lateral line

The mechanosensory lateral line system detects water movements and vibrations via neuromast stimulation. Neuromasts are the functional unit of the lateral line and are characterized by the hair cell epithelium that provides support and a cupula, which is a gelatinous coating that provides protection to the ciliary bundles to aid in directional detection of the external environment (Flock and Wersäll, 1962; Münz, 1979). Neuromasts can be grouped into two categories: (1) superficial neuromasts, which lie on the skin surface and are sensitive to changes in velocity due to hair bundle polarization being either parallel or perpendicular to the longitudinal axis of the fish; and (2) canal neuromasts, which reside below the surface of the skin protected by scales or pores and are sensitive to changes in acceleration due to the direction of hair cell polarization being adjacent to the pore openings (Schmitz et al., 2008; Coombs et al., 2014). However, it is well known that the lateral line is solely sensitive to particle motion components of water movement, thus playing some role in sound detection within close proximity of the body (Montgomery et al., 1995, 1997; Coombs and Montgomery, 1999).

Inner ear

The inner ear functions in both vestibular and auditory processes. The inner ear is composed of three semicircular canals that facilitate angular acceleration and three otolithic organs (utricle, lagena, and saccule) that are multimodal and integrate both linear acceleration and particle motion (sound) (Platt and Popper, 1981; Schellart and Popper, 1992; Popper and Fay, 1993; Popper et al., 2003a). Similar to the lateral line, the inner ear contains mechanoreceptive hair cells, which are found along the sensory epithelium of all three otolithic organs. The pattern of hair cells may depend on the fish's

environment and can be correlated with its acoustical sensitivity (Popper and Schilt, 2008). Additionally, some fishes possess specialized hearing structures (i.e. Weberian ossicles and gas-filled swim bladders), which facilitate an increase in hearing sensitivity due to the reverberation of sounds within these structures (Bridge and Haddon, 1892).

Effects of anthropogenic sound

Anthropogenic sound is any sound produced by human activity either intentionally or deliberately and can occur in the offshore, near shore and even on land within close proximity of the water (Richardson et al., 2013; Popper and Hawkins, 2019). The most common sources of anthropogenic sound globally are commercial and recreational vessels, seismic surveys, pile driving and seismic airguns (Studds and Wright, 2007; Hawkins et al., 2015; Popper and Hawkins, 2019). Some of these sound sources (i.e. vessels and pleasure crafts) may only impact the fishes within close proximity to the sound source; however, high intensity (> 170 dB re. 1μPa) sound sources, such as pile driving and seismic airguns, may potentially harm fishes at greater distances from the sound source (Slabbekoorn et al., n.d.; Popper and Hawkins, 2019).

Of mounting global concern is the rate at which human activity has expanded in and around bodies of water, which has resulted in increased anthropogenic sound pressure levels (Slabbekoorn et al., 2010; Hawkins et al., 2015; Popper and Hawkins, 2016, 2019). When considering underwater sound's ability to propagate over large distances, anthropogenic sound has the potential to significantly impact the behavior and physiology of aquatic organisms (Southall et al., 2007; Clark et al., 2009; Slabbekoorn et al., 2010; Popper and Hawkins, 2016).

Extensive research has been conducted on the impact anthropogenic sound has on population densities of terrestrial organisms (i.e. insects, birds and mammals) (Fahrig and Rytwinski, 2009; Benítez-López et al., 2010) as well as marine mammal behaviors (i.e. communication, foraging and dive behavior) (Council et al., 2000; Romano et al., 2004; Southall et al., 2007; Tyack, 2008; Ellison et al., 2012; Atkinson et al., 2015). However, fewer studies have documented the effects of anthropogenic sound on fishes, especially on their natural behaviors.

Hearing loss

Extensive hair cell damage due to exposure to anthropogenic sound can lead to decreased auditory sensitivity, which can alter a fish's behaviors and significantly impact fish's ability to detect their habitats soundscape (McCauley et al., 2003; Popper and Hastings, 2009; Ladich, 2013). However, the duration of increased auditory thresholds varies depending upon the severity of hair cell damage. If hair cells have become fatigued or some hair cell lose has occurred, temporary threshold shifts (TTS) may be observed. If repeated high intensity sound or TTS is observed , permanent threshold shifts (PTS) may occur due to significant hair cell death or damage of other auditory periphery structures (McCauley et al., 2003; Casper et al., 2013); however, PTS is generally observed among fishes within close proximity of high intensity (>170 dB re. 1 μPa) anthropogenic sound sources (Popper and Hawkins, 2019). Therefore, investigating the effects of anthropogenic sound exposure is critical to understanding at what point TTS or PTS occur.

Toadfish

Batrachoidid fishes, such as the oyster toadfish (*Opsanus tau*), Lusitanian toadfish (*Halobatrachus didactylus*) and plainfin midshipman (*Porichthys notatus*), are some of the most extensively studied vocal fishes due to their unique courtship behaviors and hardiness. Batrachoids have been used as model organisms for both behavioral and physiological studies to investigate sound vocalization (Tavolga, 1958; Gray and Winn, 1961; Fine, 1978; Brantley and Bass, 1994; Santos et al., 2000; Amorim et al., 2008; Bass and Ladich, 2008; Maruska and Mensinger, 2009), auditory capabilities (Edds-Walton and Fay, 2002; Sisneros and Bass, 2003; Vasconcelos and Ladich, 2008; Zeddies et al., 2010, 2012; Alderks and Sisneros, 2011; Vasconcelos et al., 2011; Radford and Mensinger, 2014; Maruska and Mensinger, 2015; Mensinger, 2016; Bhandiwad et al., 2017; Cardinal et al., 2018; Mensinger et al., 2019; Vetter et al., 2019) and the vestibular functions of fishes (Boyle and Highstein, 1990a; Highstein, 1992; Rabbitt et al., 1994, 1999; Mensinger et al., 1997; Boyle et al., 2001, 2018b; Highstein et al., 2005). As our understanding of these fishes has expanded, so have our technical approaches; thus, allowing researchers to develop more complex questions and expand our understanding of how fishes detect, integrate and respond to their environment.

The oyster toadfish is a vocal fish species that possess a unique vocal repertoire that includes territorial, agonistic, and in the case of males, advertisement calls (Gray and Winn, 1961; Fine, 1978; Edds-Walton and Fay, 2002; Maruska and Mensinger, 2009). In early Spring, sexually mature male toadfish establish nests in shallow waters off the eastern coast of the United States and produce an advertisement call termed a boatwhistle, to attract reproductive females to their nest for mating (Gray and Winn, 1961). The boatwhistle is produced via the contraction of sonic muscles that surround the

swim bladder and consists of a brief high amplitude broadband period (30-50 ms) followed by a longer tonal period (200-650 ms) with the boatwhistle fundamental frequency ranging from 90-250 Hz (Tavolga, 1958; Fine, 1978; Edds-Walton et al., 2002; Mensinger, 2014). Female toadfish detect, integrate, and localize these propagating boatwhistles via the inner ear and lateral line (Hastings et al., 1996; Radford and Mensinger, 2014; Maruska and Mensinger, 2015; Cardinal et al., 2018). Once female toadfish have localized the male nest, they will deposit their eggs within the nest and the males will then fertilize, fan and guard the eggs until juvenile toadfish detach and leave the nest (Mensinger et al., 2003).

Recently, researchers have investigated the afferent nerve response to male boatwhistle playbacks by conducting chronic neural recordings from the anterior lateral line nerve (Radford and Mensinger, 2014) and the utricular nerve (Maruska and Mensinger, 2015). These studies found that both systems detect playbacks of boatwhistle vocalizations and exhibit a diversity of directional sensitivity. While these studies have contributed to the physiological understanding of how toadfish are able to detect relevant stimuli (boatwhistle vocalizations), the characterization of how detection may be impacted by prolonged exposure to playbacks of boatwhistle vocalizations or anthropogenic sound and other natural behaviors, such as swimming, remains unknown.

Attempts to characterize anthropogenic sounds impact on the inner ear of fishes due to rapid global increases in anthropogenic sound levels are more frequent (Popper et al., 2003b; Slabbekoorn et al., 2010; Popper and Hawkins, 2019). Previously, anthropogenic sounds impact on hearing has been investigated in the Lusitanian toadfish;

however, experiments were conducted using speakers suspended in air rather than underwater (Vasconcelos et al., 2007; Alves et al., 2016).

During mating, toadfish can detect neighboring courting males calls (Mensinger, 2014). Additionally, toadfish vocalization rates increase throughout the mating season (Putland et al., 2018a; Van Wert and Mensinger, 2019), and can attain high intensities (~ 140 dB re. 1 μPa (Tavolga, 1958)). Therefore, the question is raised as to whether prolonged exposure to conspecific calls impacts toadfish hearing sensitivity. In Chapter 2, the auditory evoked potential recording technique was used to determine the minimum sound pressure and particle acceleration level auditory thresholds of the toadfish. Additionally, the effects of exposure to conspecific boatwhistle vocalizations and anthropogenic sound playbacks on the auditory threshold of toadfish was determined.

Additionally, both the lateral line and inner ear of fishes are sensitive to auditory (sound) and vestibular (linear acceleration) stimulation (Platt and Popper, 1981, 1981; Schellart and Popper, 1992; Popper and Fay, 1993). It has previously been postulated that for these systems to remain sensitive to external stimuli an adaptive filter or efferent modulation within higher order brain centers cancels out self-generated movements (Montgomery and Bodznick, 1994; Bell et al., 1997; Bell, 2001; Weeg et al., 2005). Although many studies have tried to investigate this hypothesis, they have primarily been conducted on immobilized or restrained fishes (Roberts and Russell, 1972; Russell and Roberts, 1972, 1974; Boyle and Highstein, 1990a; Montgomery and Bodznick, 1994; Rabbitt et al., 1995; Weeg et al., 2005). Thus, the question remains as to how external stimuli is detected in free-swimming that are exhibiting normal behaviors, such as swimming.

During predatory strikes, the toadfish lateral line remains sensitive to movement (Palmer et al., 2005). Additionally, by utilizing new recording techniques (Rogers et al., 2017) it has been shown that during swimming, the toadfish lateral line remains sensitive to vibrational stimuli (50 Hz) (Mensinger et al., 2019) without the input of efferent modulation. However, the impact that self-generated movement has on inner ear sensitivity remains unknown. Therefore, in Chapter 3, chronic neural recordings from the utricular nerve were conducted using an implantable micromanipulator, which allowed for long duration monitoring of neural activity post-implant. Experiments measured the neural response of the utricle to playbacks of conspecific vocalizations at variable speeds during assisted and free swimming to determine the effect movement has on utricular auditory sensitivity.

Chapter 2: The effect of biological and anthropogenic sound on the auditory sensitivity of Oyster Toadfish, *Opsanus tau*

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Abstract

Many aquatic organisms use vocalizations for reproductive behavior; therefore, disruption of their soundscape could adversely affect their life history. Male oyster toadfish (*Opsanus tau*) establish nests in shallow waters during spring and attract female fish with boatwhistle vocalizations. Males exhibit high nest fidelity, making them susceptible to anthropogenic sound in coastal waters, which could mask their vocalizations or reduce auditory threshold levels. Additionally, the effect of self-generated boatwhistles on toadfish auditory threshold sensitivity has yet to be addressed. To investigate the effect of sound exposure on toadfish hearing, sound pressure and particle acceleration thresholds were determined using auditory evoked potentials before and after (0, 1, 3, 6 and 9 days) exposure to 1 or 12 hrs of continuous playbacks to ship engine sound or conspecific vocalization. Exposure to boatwhistles had no significant effect on auditory thresholds. However, exposure to anthropogenic sound caused significant decreases in auditory sensitivity for at least three days, with threshold shifts up to 8 dB_{rms} re. 1 μPa SPL and 20 dB_{rms} re. 1 μPa SPL immediately following 1 and 12 hr anthropogenic exposure, respectively. Understanding the impact of self-generated and

anthropogenic sound exposure on auditory thresholds provides an insight into how soundscapes effect acoustic communication.

Introduction

Many aquatic organisms are exposed to various abiotic (i.e. waves, wind and rain), biotic (i.e. invertebrates, fishes and marine mammals vocalization) and anthropogenic (i.e. near shore construction, ship traffic and sonar) sounds. These various sound sources have the potential to interfere with, or mask, acoustic communication in the underwater soundscape (Hildebrand, 2009). Numerous investigations have shown that exposure to anthropogenic sound can negatively impact the behavior (i.e. foraging, movements, predator/prey interactions and mating) and physiology (i.e. hearing, oxygen consumption and heart rate) of aquatic organisms (Slabbekoorn et al., 2010; Radford et al., 2014; Williams et al., 2015; Shannon et al., 2016; Popper and Hawkins, 2019). While extensive research on the effects of exposure to anthropogenic sound has been conducted on marine mammals (Council et al., 2000; Holt et al., 2008; Clark et al., 2009; Ellison et al., 2012), less is known regarding anthropogenic sounds impacts on fishes (Hawkins et al., 2015). Additionally, the effect of self-generated sounds on the signaler's auditory threshold sensitivity remains largely unexplored.

In fishes, sound detection is mediated by the displacement of mechanoreceptive sensory hair cells in the lateral line and inner ear (Harris and van Bergeijk, 1962; Popper and Fay, 1973). However, similar to the auditory systems of terrestrial organisms, high intensity and/or prolonged sound exposure can result in hair cell damage, which leads to decreases in auditory sensitivity or temporary threshold shifts (TTS). For example, TTS following exposure to white noise (0.2 – 4.0 kHz; 158 dB re. 1 μPa) was observed for up to 3 days in goldfish (*Carassius auratus*) and 14 days in catfish (*Pimelodus pictus*) (Amoser and Ladich, 2003). Following repeated or intense sound exposure, permanent

threshold shifts (PTS) may occur due to significant hair cell death or damage of other auditory periphery structures (McCauley et al., 2003; Casper et al., 2013). Moving forward, it is critical to understand the sound exposure level that induces TTS or PTS and recovery durations because sound plays a vital role in the underwater communication.

The oyster toadfish (*Opsanus tau*) generates sound by contraction of sonic muscles attached to the swim bladder. Both sexes are capable of generating grunts, however, only sexually mature males produce advertisement vocalizations, termed boatwhistles (Gray and Winn, 1961; Fine, 1978; Maruska and Mensinger, 2009). In late spring to early summer, male toadfish establish nests in shallow waters along the eastern coast of the United States and acoustically attract females to their nest (Gray and Winn, 1961). The boatwhistle consists of a brief high amplitude broadband grunt (30-50 ms) followed by a regular pulsing period (200 – 650 ms), with the fundamental frequency ranging from 130 - 225 Hz (Gray and Winn, 1961; Edds-Walton et al., 2002; Van Wert and Mensinger, 2019). Male toadfish produce boatwhistles throughout the day with peak calling usually between 1900 and 0200, with some individuals producing boatwhistles every 4 s for almost the entire night (Van Wert and Mensinger, 2019). Conspecifics can detect these vocalizations via both the lateral line (Radford and Mensinger, 2014; Cardinal et al., 2018; Mensinger et al., 2019) and inner ear otoliths (Fay and Edds-Walton, 1997a, 1997b; Maruska and Mensinger, 2015), with sound intensities reaching up to 150 dB re. 1 μPa within the nest (Mensinger, 2014). However, the effect of long duration exposure to boatwhistle vocalizations on auditory sensitivity is unclear.

Toadfish require hard substrates for their nests and are often found under docks or piers, placing them in close proximity to anthropogenic sound sources. Additionally,

male toadfish exhibit high nest fidelity (Maruska and Mensinger, 2009; Mensinger, 2014; Putland et al., 2018a), which may make them more susceptible to the impact of anthropogenic sound than mobile species that can swim away from the source (Faulkner et al., 2018). Therefore, the toadfish is an excellent model to investigate the effects of anthropogenic sound. The goals of the present study were to determine toadfish baseline auditory threshold and the effect of anthropogenic sound or male boatwhistle vocalizations on toadfish auditory thresholds.

Materials and Methods

Animal husbandry

Adult toadfish (n=15; 12 male and 3 females, standard length: 26.0 ± 3.4 cm; mean ± s.d.) were obtained from the Marine Biological Laboratory in Woods Hole, MA and housed at the University of Minnesota Duluth. Toadfish were maintained in 515 L recirculating tanks (120 cm length x 95 cm width x 40 cm water depth; Miller Manufacturing, Eagan, MN) filled with artificial saltwater (35 PSU, Instant Ocean, Blacksburg, VA) that was mechanically, chemically, and biologically filtered (1500 Penn-Plax Cascade^{TM} filters) and maintained at 18.0 ± 0.5 °C. All experimental procedures conformed to institutional animal care protocols.

Acoustic evoked potential (AEP) recordings

Acoustic evoked potential (AEP) recordings were conducted between November 2018 and February 2019. AEPs were performed in a 375 L cylindrical fiberglass experimental tank (90 cm diameter x 60 cm water depth) placed on a 1 cm thick rubber mat to dampen vibrations. The experimental tank was enclosed within a galvanized angle iron frame (110 x 125 x 180 cm) covered on three sides and the top with FOAMULAR

Insulation Sheathing (2.54 cm thick; Owens Corning, Toledo, OH) to reduce background sound.

Toadfish were anesthetized by immersion in 0.005% tricaine solution and immobilized with an intramuscular injection of 0.01% pancuronium bromide (600 μg kg^{-1}). Toadfish were suspended in a mesh sling above the experimental tank using an adjustable arm boom stand (Omano Microscopes, China). Two insulated stainless-steel electrodes (Rochester Electro-Medical Inc., Tampa, FL) were subcutaneously inserted into the midline of the toadfish head. The reference electrode was positioned 5 mm from the rostrum and centered between the nares, while the recording electrode was inserted along the dorsal midline directly above the brainstem approximately 6 mm anterior to the posterior end of the cranium. For serial testing, a small bolus of cyanoacrylate gel was placed on the epidermis of the toadfish immediately posterior to the recording electrode to ensure that the electrode was inserted in the same position in subsequent testing. Toadfish were submerged with their dorsal surface 5 cm below the surface and their ventral surface 40 cm above the underwater speaker. Electrodes were connected to a headstage (gain = 10x) that connected to an extracellular differential amplifier (gain = 100x; Dagan, Minneapolis, MN). The signal was filtered (band pass 0.03 to 3 kHz) and recorded with Spike2 software (Cambridge Electronic Design Ltd, Version 8) using a custom Spike2 script (Cambridge Electronic Design; Cambridge, UK) and monitored on a portable computer.

Initial AEP testing revealed that toadfish (n=5) did not respond to pure tones > 500 Hz, therefore subsequent AEP recordings were conducted in response to 100, 120, 140, 160, 180, 200, 220, 240, 260, 280, 300, 350, 400 and 500 Hz pure tone bursts (50

ms, 500 repetitions, 3 ms delay). AEP waveforms were verified qualitatively by AEP visual inspection and quantifiably by fast Fourier transform power spectrum analysis (FFT, Hamming Window = 1024), with the minimum FFT level ≥ 0.001 μV (Vetter et al., 2018; Nissen et al., 2019).

After baseline auditory thresholds were determined, individual toadfish were placed in a 375 L trough sound exposure tank (50 cm water depth, top: 125 cm length x 75 cm maximum width, bottom: 100 cm x 50 cm; Rubbermaid Commercial Products, Winchester, VA). An underwater speaker (Clark Synthesis AQ-339; Littleton, CO) was submerged at one end of the tank and was operated by an amplifier (Bosch Plena; Farmington Hills, MI) and Roland 4-channel portable recorder (R-44; Roland Corporation; Hamamatsu, Japan). A mesh barrier was placed at the opposite end to restrict toadfish to 50 – 80 cm from the speaker (Figure 1a). Toadfish (n = 5 fish/treatment) received one of three treatments: a 12 hr boatwhistle playback consisting of an underwater sound recording from an individual male toadfish boatwhistle vocalization (425 ms duration; fundamental frequency: 180 Hz; playback frequency: 0.25 Hz), or a 1 or 12 hr playback of a broadband anthropogenic sound recorded underwater from an idling 15 m research vessel (Detroit diesel engine; power output: 7 – 1193 kW 12V-71; single screw; broadband frequency range: 30 – 12000 Hz; 2 min duration). Following sound exposure, toadfish were tested immediately (0 day), and then 1, 3,6 and 9 days after exposure. (Figure 1b, c, d, & e).

Sound exposure

Sound pressure levels (SPL; dB_{rms} re. 1 μPa) and particle acceleration levels (PAL; dB_{rms} re. 1 ms^{-2}) were determined approximately 7.5 cm from the bottom of the

experimental tank at 18 locations within the end containing the toadfish. Sound pressure levels were determined using a calibrated hydrophone (HTI-96-MIN; High Tech Inc., Long Beach, MS; open circuit voltage (OCV) with preamp battery = -165 dB re. 1 V/μPa), while particle acceleration levels were calculated using a neutrally buoyant waterproofed triaxial accelerometer (Model: W356A12/NC; PCB Piezotronics, Depew, NY; Sensitivity: X = 10.47 mV/ms^{-2}; Y = 10.35 mV/ms^{-2}; Z = 10.29 mV/ms^{-2}) connected to a signal conditioner (Model: 482C; PCB Piezotronics, Depew, NY). All data was recorded using PowerLab data acquisition system and analyzed offline as the voltage root mean square (rms) using LabChart software (Version 8). V_{rms} values measured with the hydrophone were converted to dB and then corrected for the open circuit voltage (Eq. 1). Sound pressure level for boatwhistles and anthropogenic sound were maintained at approximately 150 dB re 1 μPa between 80 – 550 Hz within the toadfish end.

Eq. 1: $dB_{rms}\ re.1\ \mu Pa = \ 20\ Log_{10}(V_{rms}) - OCV$

V_{rms} values for each axis (X, Y and Z) of the particle accelerometer were calibrated to the sensitivity of the accelerometer and used to calculate the magnitude of particle acceleration in dB scale (Eq. 2) (Vetter et al., 2018, 2019; Nissen et al., 2019).

Eq. 2: $dB_{rms}\ re.1\ ms^{-2} = \ 20\ Log_{10}(\sqrt{X^2 + \ Y^2 + \ Z^2})$

All calculations for sound pressure and particle acceleration levels were performed within a custom Matlab software (Version2017a) script.

Particle acceleration thresholds

Particle acceleration thresholds were determined via a waterproofed triaxial accelerometer that was placed within the AEP experimental tank at the position of the toadfish head during testing. For a given frequency, particle acceleration measurements

were made across the corresponding sound intensity range. Using a custom Matlab (Version 2017a) script, particle acceleration measurements (V_{rms}) for each axis *(X, Y* and *Z*) were corrected for the sensitivity of the accelerometer (Figure 2) and particle acceleration level thresholds were determined (Eq. 2).

Statistical analysis

To determine the effects of sound exposure and recovery period on the auditory threshold of toadfish, a two-way repeated measure analysis of variance (ANOVA) with frequency (Hz) and time (baseline, 0, 1, 3, 6 or 9 days post-exposure) as factors and threshold measurements as the dependent variable was performed. A Holm-Sidak post-hoc test determined the significance of each frequency threshold shift from baseline (α = 0.05). All statistical analyses were performed using SigmaPlot software (Version 13).

Results

Sound exposure

The mean background PAL and SPL measured within the exposure tank in the toadfish area was -48.4 ± 1.7 dB_{rms} re. 1 ms^{-2} PAL and 102.5 ± 0.9 dB_{rms} re. 1 μPa SPL, respectively (Figure 3a). PAL and SPL increased during boatwhistle playbacks, to -3.8 ± 1.5 dB_{rms} re. 1 ms^{-2} PAL and 151.9 ± 0.9 dB_{rms} re. 1 μPa SPL (Figure 3b), while anthropogenic playbacks attained levels up to -4.1 ± 2.9 dB_{rms} re. 1 ms^{-2} PAL and 152.1 ± 1.2 dB_{rms} re. 1 μPa SPL (Figure 3c).

Auditory evoked potentials

Toadfish (n =15, 12 males and 3 females) responded to all tested frequencies between 100 and 500 Hz. Figure 4 displays two representative AEP waveforms and FFT analyses in response to 220 and 400 Hz, respectively. Baseline SPL (dB_{rms} re. 1 μPa) and

PAL (dB_{rms} re. 1 ms^{-2}) auditory evoked potential (AEP) responses were observed at all frequencies tested (Figure 5). Fish displayed greatest auditory threshold sensitivity at 100 Hz (SPL: 116.2 ± 6.1 dB_{rms} re. 1 µPa; PAL: -49.1 ± 6.6 dB_{rms} re. 1 ms^{-2}) and 120 Hz (SPL: 116.3 ± 6.4 dB_{rms} re. 1 µPa; PAL: -47.2 ± 6.2 dB_{rms} re. 1 ms^{-2}). Auditory thresholds increased up to 500 Hz (SPL: 145.1 ± 3.6 dB_{rms} re. 1 µPa; PAL: -17.9 ± 3.9 dB_{rms} re. 1 ms^{-2}) (Figure 5). AEPs above 500 Hz were not detectable at the maximum sound pressure levels presented (150 dB re. 1 µPa).

Anthropogenic playbacks

Following 1 hr anthropogenic sound exposure (n=5, 4 males and 1 female), temporary SPL and PAL auditory threshold shifts were observed in all fish between 100 – 400 Hz (Figure 6) with 2 fish showing no response to 500 Hz. To determine if auditory threshold levels significantly shifted from baseline, a two-way repeated measures ANOVA was conducted between 100 and 400 Hz. Significant threshold shifts at 500 Hz, were determined by removing day 0 from analysis and only post-hoc values at 500 Hz were used to determine significant threshold shifts across 1, 3, 6 and 9 days post-exposure.

Significant auditory threshold shifts from baseline (two-way repeated measures ANOVA, d.f. = 5, F = 8.58, $p < 0.001$) were observed only across PAL auditory threshold levels (Table 1). Significant PAL auditory threshold frequency shifts were observed at 100, 160, 180, 200 and 300 Hz (Holm-Sidak, $p < 0.05$) immediately following (0 day) anthropogenic sound exposure. At 1 day, significant frequency shifts were sustained at 100, 120, 160, 180, 200, 300, and 350 Hz. By 3 days post-anthropogenic sound exposure significant frequency shifts from baseline were observed

at 160, 180, and 300 Hz. At 6 days post-exposure, there were no significant difference between pre- and post-exposure PAL (Table 1, Figure 6).

Following 12 hr anthropogenic sound exposure temporary SPL and PAL threshold shifts were observed across all frequencies (0 – 400 Hz), with 4 fish exhibiting no response at 500 Hz (n=5, 4 males and 1 female; Figure 7). Significant shifts across auditory thresholds (100 – 400 Hz) were observed for both 12 hr anthropogenic SPL (two-way repeated measures ANOVA, d.f. = 5, F = 12.34, $p < 0.001$) and PAL threshold curves (two-way repeated measures ANOVA, d.f. = 5, F = 12.51, $p < 0.001$). Recovery of auditory thresholds were observed as soon as 1 day, however significant frequency shifts (Holm-Sidak, $p < 0.05$) were sustained for SPL thresholds at 140, 160, 200 and 260 Hz and for PAL thresholds at 100, 140, 160, 200 and 260 Hz. At 3 days, significant frequency shifts (Holm-Sidak, $p < 0.05$) were observed at 100, 140, 160, 200, and 240 Hz for both SPL and PAL auditory thresholds. By 6 days post-exposure, significant SPL and PAL threshold and frequency shifts were no longer observed (Holm-Sidak, SPL: $p = 0.26$; PAL: $p = 0.26$, Table 2 & 3, Figure 7).

Boatwhistle playbacks

Following exposure to 12 hrs of boatwhistle playbacks (180 Hz fundamental frequency; 425 ms duration; 0.25 Hz playback frequency), toadfish (n = 5; 4 males and 1 female) auditory thresholds displayed an increased auditory sensitivity (~ 8 dB_{rms} re. 1 µPa SPL or 6 dB_{rms} re. 1 ms^{-2} PAL) to frequencies between 300 and 500 Hz compared to baseline auditory thresholds (Figure 8). However, no significant auditory threshold (SPL and PAL) shifts were observed when compared to baseline levels (two-way repeated

measures ANOVA, d.f. = 5, SPL: F = 2.684, $p = 0.052$, PAL: F = 1.393, $p = 0.27$) (Figure 8).

Discussion

Toadfish auditory thresholds ranged from 100 to 500 Hz, with greatest auditory threshold sensitivity to frequencies within the fundamental frequency range (100 – 225 Hz) of toadfish vocalizations. Additionally, toadfish were capable of retaining auditory sensitivity when exposed to playbacks of conspecific vocalizations; however, significant threshold shifts were observed following exposure to anthropogenic sound playbacks.

Previous studies had indicated a slightly extended toadfish frequency sensitivity range up to 800 Hz (Yan et al., 2000), while a classic conditioning study determining thresholds up to 600 Hz (Fish and Offutt, 1972). However, these studies were complicated by the use of an aerial speaker and only measured sound pressure (Fish and Offutt, 1972; Yan et al., 2000). The underwater sound presentation, which included measuring the particle motion as it is the component of underwater sound that all fish are capable of detecting (Popper and Fay, 2011), may provide a more accurate analysis of the toadfish hearing range. The differences between SPL and PAL auditory threshold curves show the necessity of measuring both parameters as SPL analysis alone would have underestimated the impacts of anthropogenic sound exposure.

The effects of anthropogenic sound on fishes is just beginning to be understood and it is important to determine baseline auditory threshold curves to assess impact. Behavioral experiments have shown that anthropogenic sound can impact reproductive behaviors (Bruintjes and Radford, 2013; Ladich, 2013), predator/prey interactions (Voellmy et al., 2014a, 2014b; Simpson et al., 2015), larval fish orientation and

settlement (Radford et al., 2011; Holles et al., 2013) and schooling (Sarà et al., 2007; Herbert-Read et al., 2017). Physiological experiments have determined that anthropogenic sound increases stress (Santulli et al., 1999; Wysocki et al., 2006; Sierra-Flores et al., 2015), damages auditory structures (McCauley et al., 2003; Smith et al., 2006) and induces temporary auditory threshold shifts (Smith et al., 2004; Popper et al., 2005; Vasconcelos et al., 2007; Nissen et al., 2019).

Within coastal recreational waterways, passive acoustic monitoring studies have observed that anthropogenic sound pressure levels produced by recreational vessels do not often surpass 120 dB re. 1 μPa (Haviland-Howell et al., 2007; Erbe, 2013; Marley et al., 2017). However, many toadfish reside in areas frequented by commercial vessels, and in the coastal waters of Massachusetts, anthropogenic sound pressure levels produced by research vessels, tug boats and private yachts can exceed 160 dB re. 1 μPa (71 – 224 Hz) (Hatch et al., 2012), and have the potential to mask boatwhistle vocalizations. To simulate anthropogenic sound that toadfish encounter, source level underwater recordings were taken from the Marine Biological Laboratory's RV Gemma (15 m) while it idled at its dock in Eel Pond, Woods Hole, MA, where a reproducing toadfish population resides (Mackiewicz et al., In review; Putland et al., 2018a; Van Wert and Mensinger, 2019). In this study, even short duration (1 hr) anthropogenic exposure led to significant auditory threshold shifts up to 8 dB_{rms} re. 1 μPa SPL or 9 dB_{rms} re. 1 ms^{-2} PAL immediately after sound exposure, with sustained exposure (12 hrs) resulting in larger shifts up to 20 dB_{rms} re. 1 μPa SPL or 22 dB_{rms} re. 1 ms^{-2} PAL and frequency shifts persisting for at least 3 days. Fish in both treatments recovered by day six, indicating the effects was transient, however repeated exposure may lead to permanent threshold shifts.

The behavioral impacts that decreased auditory sensitivity may have on toadfish have yet to be determined; however, reproductive success relies on the female detecting male vocalizations (Fish, 1972). The proclivity of toadfish for hard substrates often places them near docks and pilings with high boat traffic and human activity that can impact toadfish auditory sensitivity, Additionally, male toadfish exhibit high nest fidelity (Maruska and Mensinger, 2009; Mensinger, 2014; Putland et al., 2018a), which makes them susceptible to anthropogenic sounds as they are unlike to leave the area. Therefore, the decreased auditory sensitivity following anthropogenic sound exposure could negatively impact toadfish reproductive success.

Toadfish populations are also exposed to natural ambient sound including the vocalizations of conspecifics. Field recordings of toadfish vocalizations highlight that individuals can produce sound intensities ranging from 130 – 140 dB re. 1µPa @ 1m (Tavolga, 1971) with individuals vocalizing up to 15 times per minute during the night (Ricci et al., 2017; Putland et al., 2018a; Van Wert and Mensinger, 2019). At close proximity (< 20cm), within the nest, boatwhistle vocalizations also reverberate and approach source level sound intensities up to 150 dB re. 1 µPa (Mensinger, 2014). Additionally, boatwhistle vocalizations can be interspersed by grunts, which target conspecifics vocalizations, resulting in sustained frequent and high intensity sound throughout the night (Maruska and Mensinger, 2009; Mensinger, 2014). However, significant shifts in auditory thresholds were not observed following boatwhistle playbacks. Since toadfish were tested outside their mating season, it is possible toadfish were not as sensitive to sound and were not affected by the playbacks. For example, the closely related plainfin midshipman (*Porichthys notatus)* displays seasonal auditory

plasticity, with increased auditory sensitivity occurring during the mating season (Sisneros and Bass, 2003). The boatwhistles also have a narrow frequency range compared to the anthropogenic sound and the AEPs may have missed finer scale sensitivity changes. Additionally, it has been postulated that toadfish have an adaptive filter or a mechanism to cancel out self-generated noise and allow for sustained auditory sensitivity similar to *P. notatus* (Weeg et al., 2005). However, if this mechanism exists, it is more likely utilized during the production of self-generated sounds as the adaptive filter needs to be activated prior to vocalizing and would not become activated from sounds produced by conspecifics or speakers. Yet, it remains possible that the consistent inter-call interval (4 s) allowed the toadfish to anticipate the next call and activate the adaptive filter mechanisms.

The AEP technique allowed for minimally invasive monitoring and sequential testing of fish auditory sensitivity following exposure to varying sound treatments. However, caution should always be used in interpreting the data. Although the AEP tank (375 L) is larger than many other AEPs set ups (Vasconcelos et al., 2007; Ladich and Schulz-Mirbach, 2013), it is still a relatively small tank and sound reverberations or echoes can influence results. The sound exposure experiments tried to alleviate some of the complications of small tanks by limiting the toadfish to a specific area allowing for relatively uniform sound pressure and particle acceleration levels. Additionally, it must be noted that AEPs represent a gross threshold response and behavioral experiments or single unit recording could potential reveal greater effects on the impact of sound exposure.

In conclusion, toadfish display auditory thresholds that encompass the frequency range of vocalizations emitted by toadfish. Additionally, exposure to even short durations (1 hr) of high intensity (~150 dB re. 1 μPa) anthropogenic sound is capable of causing significant temporary threshold shifts that are sustained for at least 3 days post-exposure. These significant threshold shifts may be enough to impact female sound source localization and the reproductive success of these fish.

Acknowledgments: The authors would like to acknowledge the Marine Resources Center staff at the Marine Biological Laboratory for providing toadfish. Funding was provided by National Science Foundation Grants IOS 1354745 and DBI 1359230 & 1659604 to AFM, and by the National Science Foundation Graduate Research Fellowship Program under grant DGE 1804377 to LSR.

Figures

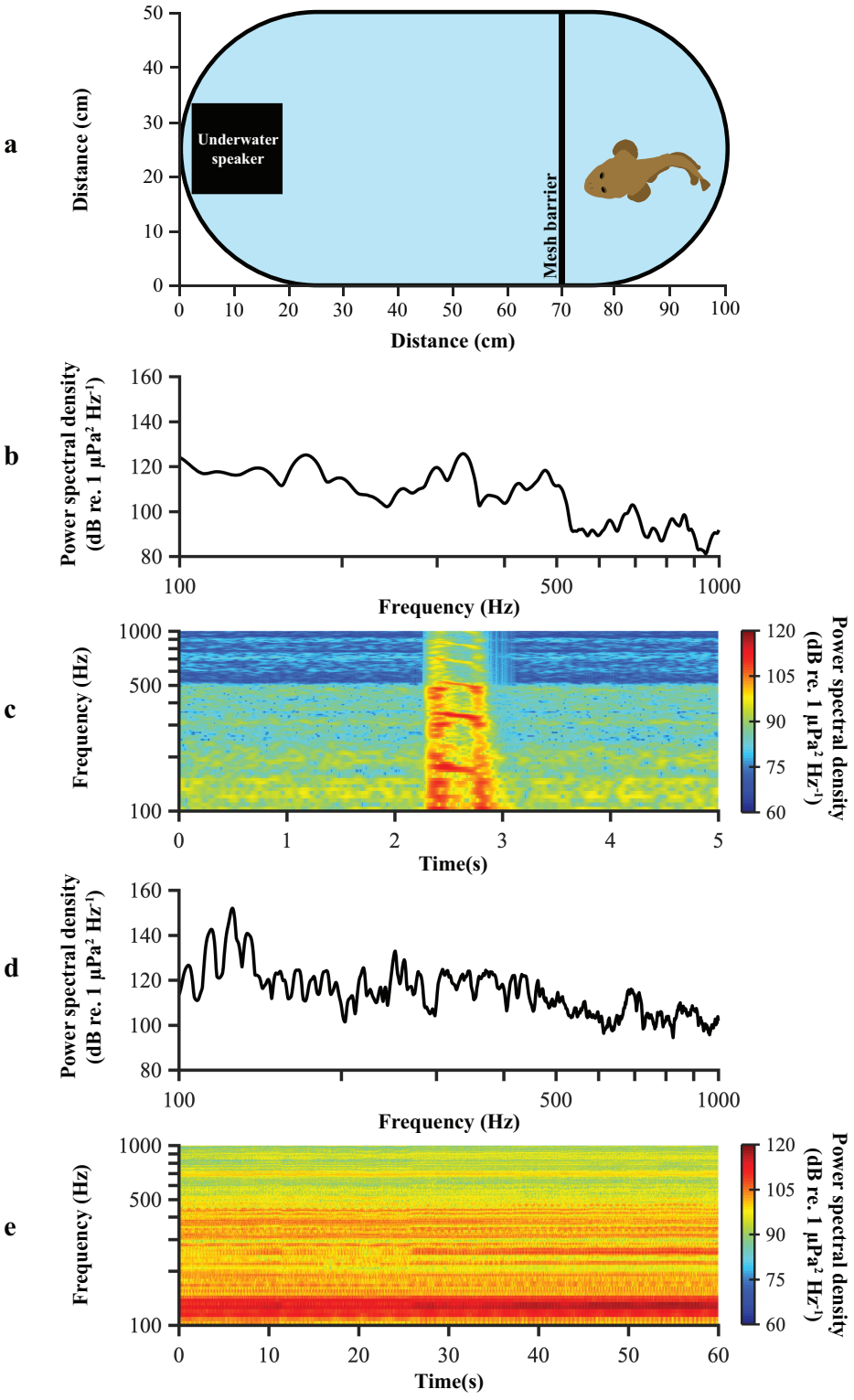
a
Underwater speaker
Mesh barrier
Distance (cm)
Distance (cm)
b
Power spectral density (dB re. 1 µPa² Hz⁻¹)
Frequency (Hz)
c
Frequency (Hz)
Time(s)
Power spectral density (dB re. 1 µPa² Hz⁻¹)
d
Power spectral density (dB re. 1 µPa² Hz⁻¹)
Frequency (Hz)
e
Frequency (Hz)
Time(s)
Power spectral density (dB re. 1 µPa² Hz⁻¹)

Figure 1: Sound exposure. (a) Top view schematic of sound exposure tank showing speaker and toadfish position. The mesh barrier maintained toadfish a minimum distance of 50 cm from the speaker. Playback power spectral density curves and spectrograms of the (b & c) boatwhistle vocalization (180 Hz fundamental frequency; 425 ms duration; 0.25 Hz playback frequency; 150 dB re. 1μPa) and (d & e) anthropogenic sound (150 dB re. 1μPa). Note the varying time scales of C and E.

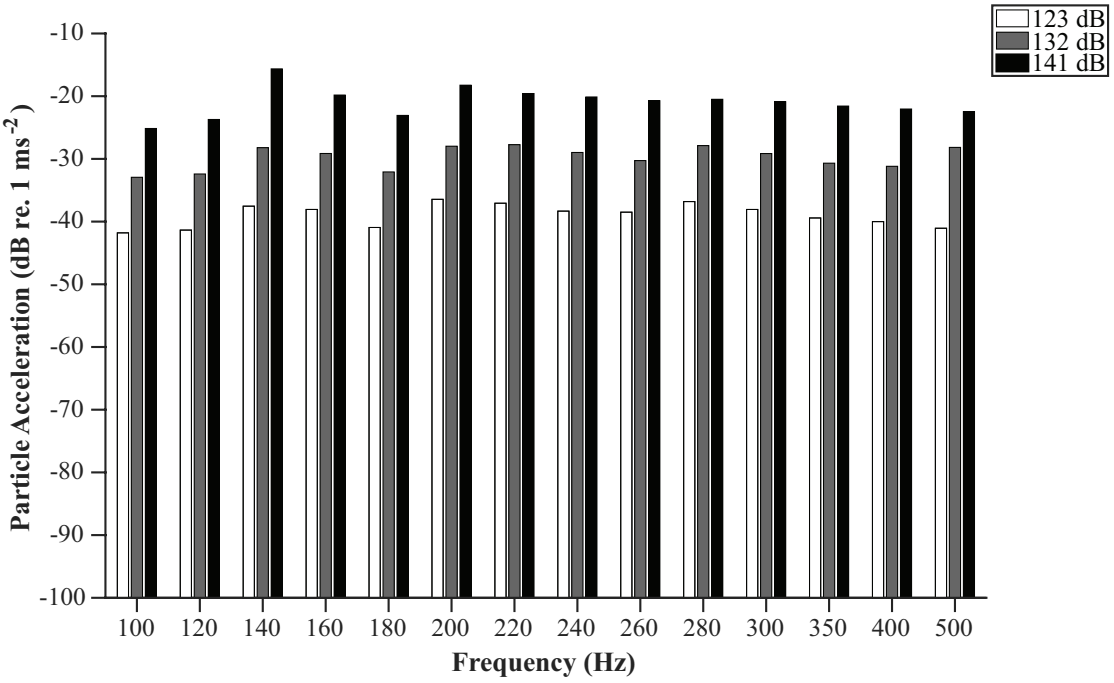

Figure 2: Particle acceleration levels (dB re. 1 ms^{-2}) across all frequencies used during AEP testing for three representative sound pressure levels (123 (white), 132 (grey) and 141 (black) dB re. 1 μPa). Particle acceleration was measured using a triaxial accelerometer positioned at the level of the fish head.

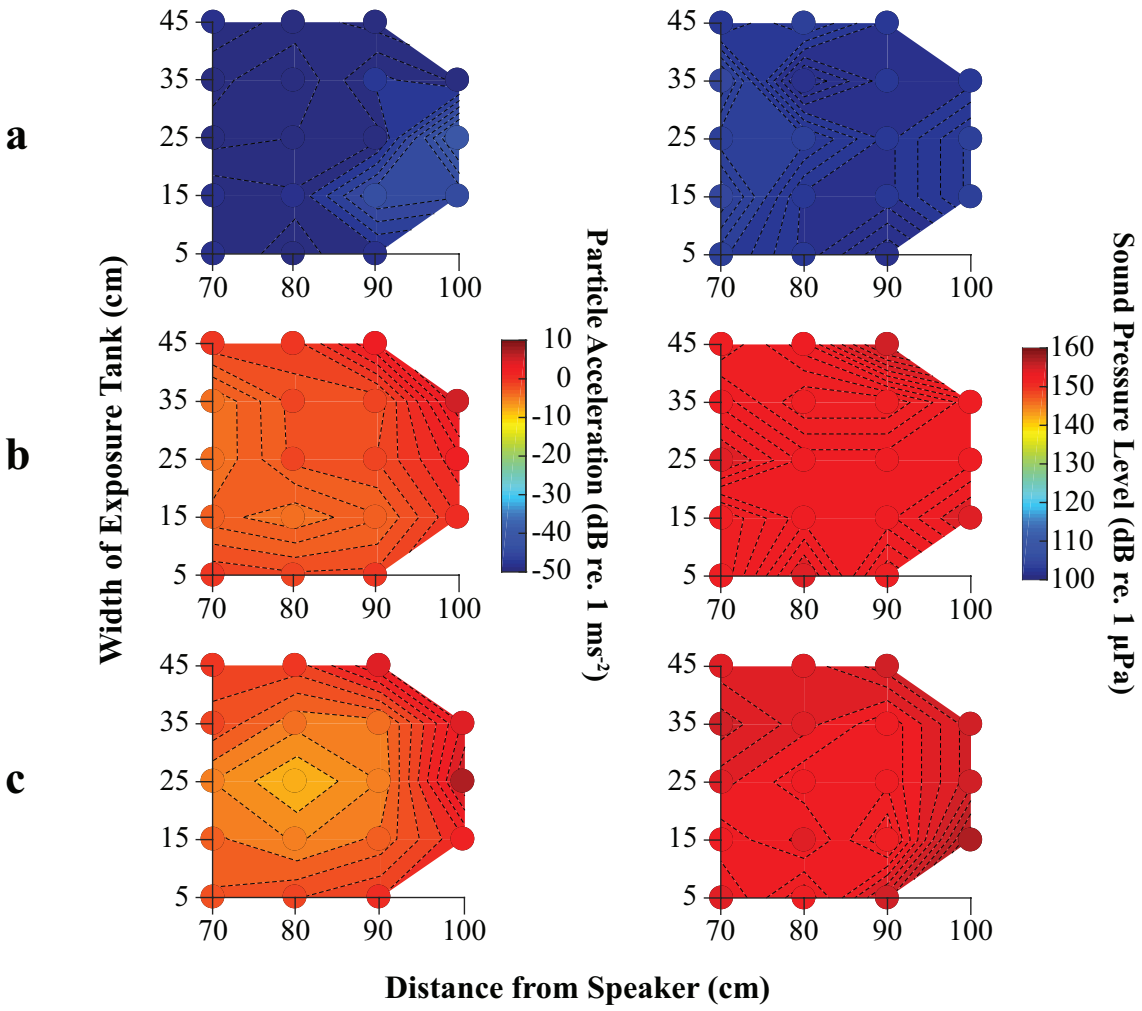

Figure 3: Sound maps in the toadfish end of the tank for particle acceleration (dB re. 1 ms^{-2}; left) and sound pressure level (dB re. 1 µPa; right) for (a) background, (b) boatwhistle playbacks and (c) anthropogenic sound playbacks. The maps were constructed from the average measurements (n=5) at 18 locations 7.5 cm from the bottom within the toadfish area.

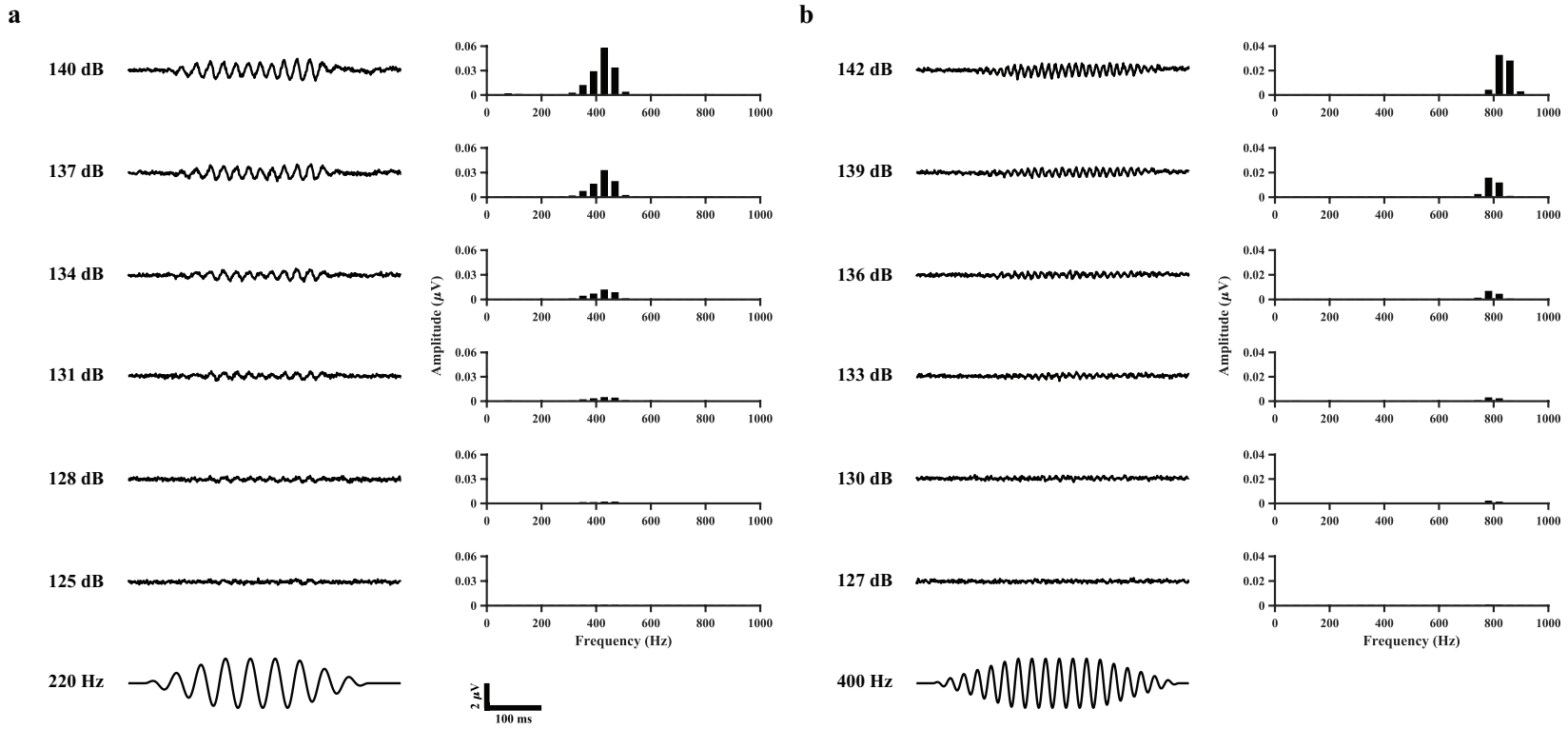

Figure 4: Auditory evoked potential (AEP) response to (a) 220 Hz and (b) 400 Hz. Each panel displays the average AEP trace (500 repetitions) for the indicated sound pressure level (dB re. 1 μPa) on the left and the fast Fourier transformation (FFT) analysis on the right. AEP thresholds were determined to be 128 and 130 dB re. 1 μPa for 220 and 400 Hz, respectively.

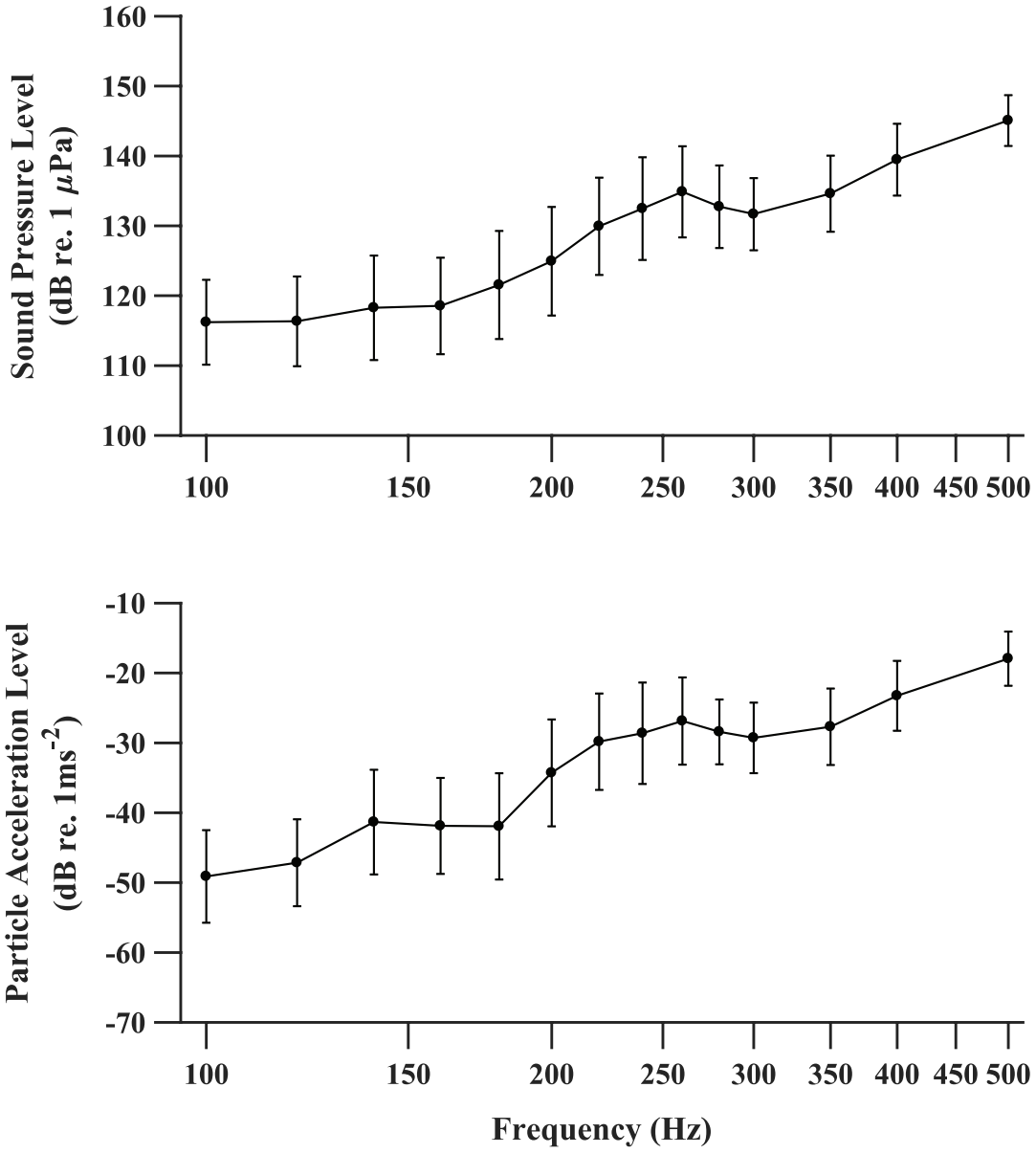

Figure 5: The mean minimum baseline sound pressure (dB re. 1 μPa; top) and particle acceleration levels (dB re. 1 ms^{-2}; bottom) needed to evoke an AEP response is plotted versus sound frequency (Hz). Data is plotted as mean ±1 s.d. (n=15).

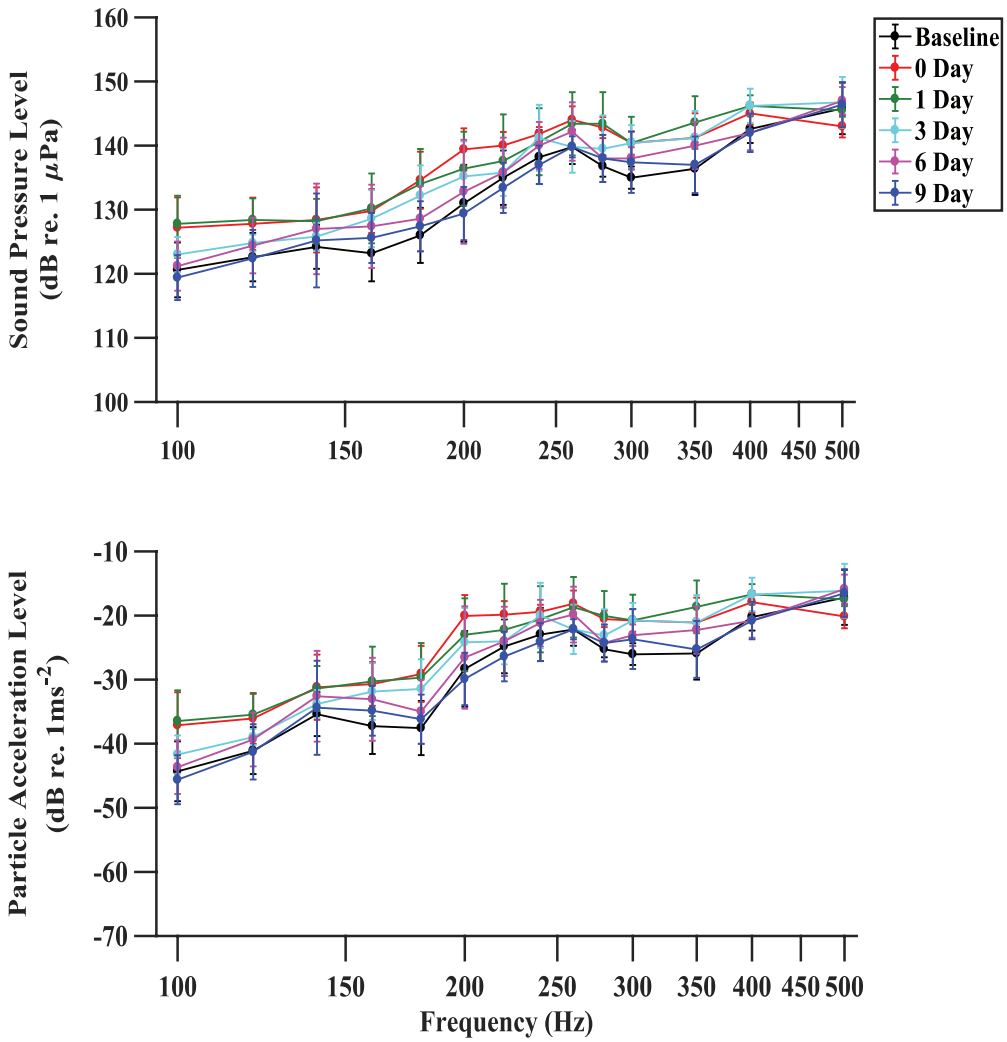

Figure 6: Auditory threshold tuning curves for data related to exposure to 1 hr of continuous broadband anthropogenic sound (Frequency range: 30 – 12000 Hz; sound pressure ~150 dB re. 1 μPa (80 – 550 Hz)). The minimum sound pressure (dB re. 1 μPa; top) and particle acceleration (dB re. 1 ms^{-2}; bottom) levels needed to evoke an AEP response is plotted versus frequency (Hz). Colors represent the pre-exposure (baseline, black) and post-exposure (day 0, red; day ,1 green; day 3, light blue; day 6, pink; day 9, blue) to 1 hr of continuous broadband anthropogenic sound. Data is plotted as mean ± 1 s.d. (n=5).

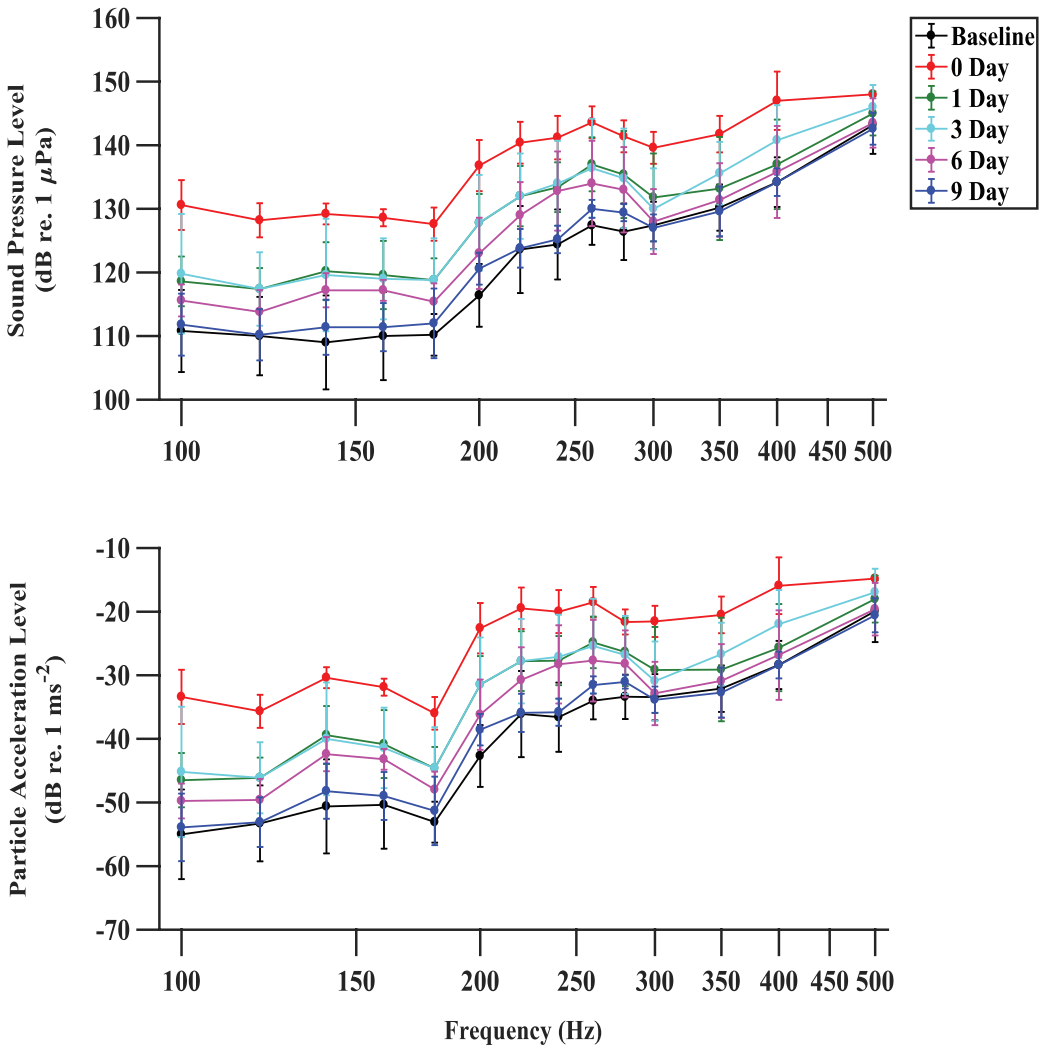

Figure 7: Auditory threshold tuning curves for data related to exposure to 12 hr of continuous broadband anthropogenic sound (Frequency range: 30 – 12000 Hz; sound pressure ~150 dB re. 1 μPa (80 – 550 Hz)). The minimum sound pressure (dB re. 1 μPa; top) and particle acceleration (dB re. 1 ms^{-2}; bottom) levels needed to evoke an AEP response is plotted versus frequency (Hz). Colors represent the pre-exposure (baseline, black) and post-exposure (day 0, red; day ,1 green; day 3, light blue; day 6, pink; day 9, blue) to 12 hrs of continuous broadband anthropogenic sound. Data is plotted as mean ± 1 s.d. (n=5).

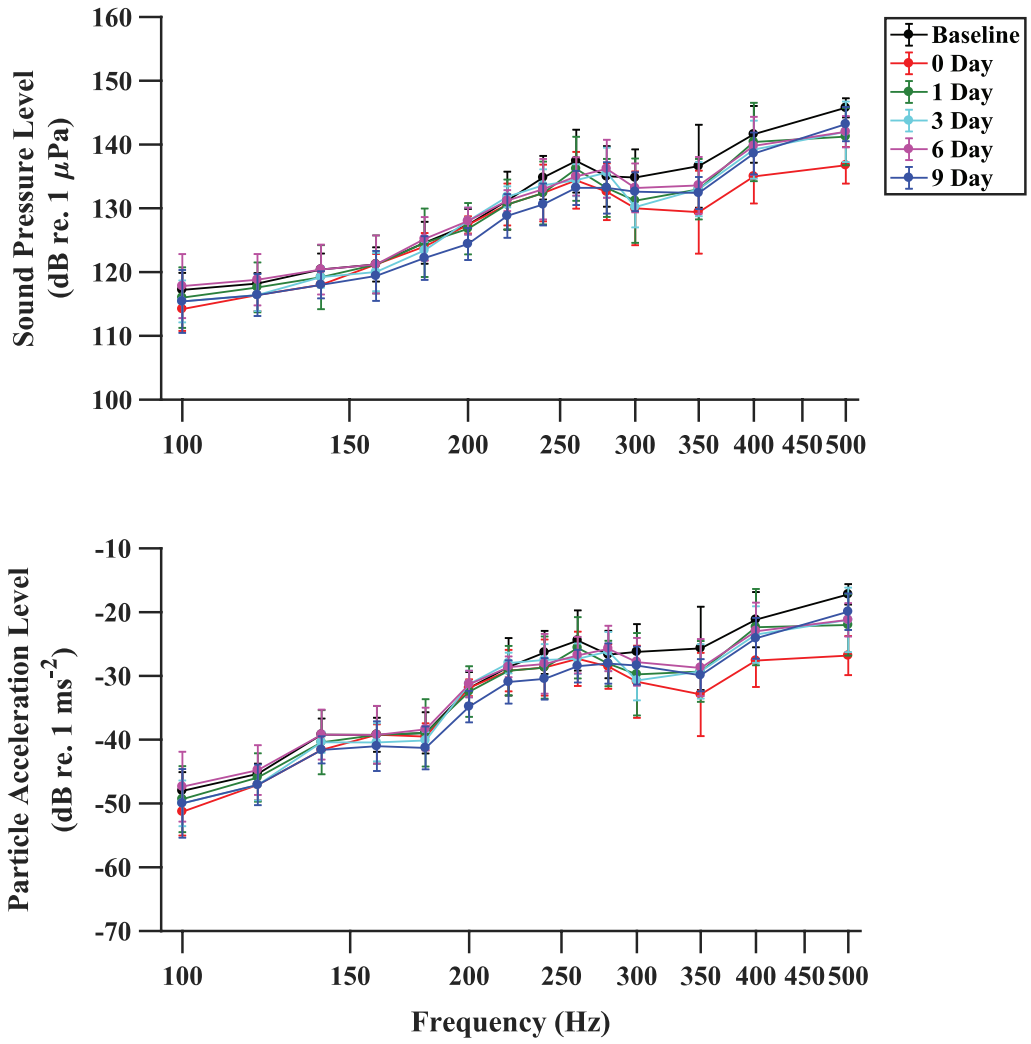

Figure 8: Auditory threshold tuning curves for data related to exposure to 12 hr of boatwhistle playbacks (425 ms duration; 180 Hz fundamental frequency, 0.25 Hz playback frequency, sound pressure ~150 dB re. 1 μPa (80 – 550 Hz)). The minimum sound pressure (dB re. 1 μPa; top) and particle acceleration (dB re. 1 ms^{-2}; bottom) levels needed to evoke an AEP response is plotted versus frequency (Hz). Colors represent the pre-exposure (baseline, black) and post-exposure (day 0, red; day ,1 green; day 3, light blue; day 6, pink; day 9, blue) to 12 hrs of boatwhistle playbacks. Data is plotted as mean ± 1 s.d. (n=5).

Table 1: Particle acceleration level (dB re. 1 ms^{-2}) threshold shifts during serial testing after exposure to 1 hr anthropogenic sound. Table values show significance levels in comparison to baseline (Holm-Sidak, $p < 0.05$). NS = not significant.

	Frequency (Hz)													
	100	**120**	**140**	**160**	**180**	**200**	**220**	**240**	**260**	**280**	**300**	**350**	**400**	**500**
0 day	P<0.001	ns	ns	P=0.004	P<0.001	P<0.001	ns	ns	ns	ns	P=0.045	ns	ns	ns
1 day	P<0.001	P=0.025	ns	P=0.002	P<0.001	P=0.03	ns	ns	ns	ns	P=0.041	P=0.001	ns	ns
3 day	ns	ns	ns	P=0.035	P=0.008	ns	ns	ns	ns	ns	P=0.048	ns	ns	ns
6 & 9 day	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 2: Sound pressure level (dB re. 1 μPa) threshold shifts during serial testing after exposure to 12 hr anthropogenic sound. Table values show significance levels in comparison to baseline (Holm-Sidak, $p < 0.05$). NS = not significant.

	Frequency (Hz)													
	100	**120**	**140**	**160**	**180**	**200**	**220**	**240**	**260**	**280**	**300**	**350**	**400**	**500**
0 day	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P=0.003	P=0.001	P<0.001	N/A
1 day	ns	ns	P=0.007	P=0.033	ns	P=0.01	ns	ns	P=0.036	ns	ns	ns	ns	ns
3 day	P=0.048	ns	P=0.012	P=0.043	ns	P=0.006	ns	P=0.036	ns	ns	ns	ns	ns	ns
6 & 9 day	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Table 3: Particle acceleration level (dB re. 1 ms^{-2}) threshold shifts during serial testing after exposure to 12 hr anthropogenic sound. Table values show significance levels in comparison to baseline (Holm-Sidak, $p < 0.05$). NS = not significant.

	Frequency (Hz)													
	100	**120**	**140**	**160**	**180**	**200**	**220**	**240**	**260**	**280**	**300**	**350**	**400**	**500**
0 day	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P<0.001	P=0.004	P=0.003	P<0.001	P<0.001	N/A
1 day	P=0.049	ns	P=0.005	P=0.028	ns	P=0.005	ns	ns	P=0.043	ns	ns	ns	ns	ns
3 day	P=0.018	ns	P=0.009	P=0.041	ns	P=0.005	ns	P=0.033	ns	ns	ns	ns	ns	ns
6 & 9 day	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Chapter 3: The effects of multimodal input on utricular sensitivity in free-swimming toadfish, *Opsanus tau*

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Abstract

The inner ear of fishes is composed of three paired otolithic end organs (saccule, utricle and lagena), which encode auditory and vestibular stimuli. To determine the effects of vestibular (movement) and auditory (pure tones or conspecific vocalizations) input, microwire electrodes were implanted using a 3D printed micromanipulator into the utricular nerve of oyster toadfish, *Opsanus tau*. Fish swam freely (velocity: 3.5 – 18.6 cm s^{-1}; acceleration: 0.8 – 24.4 cm s^{-2}) or were moved at variable speeds while affixed to a sled (velocity: 4.0 – 12.2 cm s^{-1}; acceleration: 0.3 – 2.0 cm s^{-2}). All utricular afferents responded to movement and were characterized as either phasic or phasic-tonic fibers. Afferents remained sensitive to pure tones (125 – 200 Hz) and playbacks of conspecific boatwhistles (180 Hz fundamental frequency) throughout movement. This research is the first to simultaneously investigate the effects of multimodal input to the utricle in free-swimming fish.

Introduction

The vertebrate inner ear functions to maintain equilibrium and detecting sound. Throughout evolution, the semicircular canals of the inner ear have remained highly conserved functioning primarily as vestibular organs; however, the otolithic organs have evolved in concert with the change from an aquatic to a terrestrial environment (Baird, 1974; Wever, 1974; Manley and Clack, 2004; Manley, 2012). In teleosts, the inner ear consists of three semicircular canals that detect angular motion and three otolithic end organs (saccule, utricle and lagena), which are multimodal and encode linear acceleration (vestibular) and sound detection (auditory) (Platt and Popper, 1981; Schellart and Popper, 1992; Popper and Fay, 1993). As vertebrates began to colonize land, the inner ear evolved to function in an airborne environment, with pronounced separation between auditory and vestibular functions (Manley, 1972; Clack, 2002). These included tympanums (amphibians and reptiles), external ear openings (mammals and birds) and middle ear ossicles to transmit airborne sounds into the fluid-filled canals of the inner ear (von Bekesy, 1960; Wever, 1974; Clack, 2016). In modern mammals and birds, the cochlea and avian cochlear duct, respectively, evolved as hearing organs, with vestibular functions mediated via the semicircular canals and otoliths.

In teleosts, separate hearing organs did not evolve; therefore, the otoliths remain multimodal and encode both vestibular and auditory input. The saccule has been considered the primary auditory end organ, However, the utricle detects and is directionally sensitive to sound (Fay, 1984; Lu et al., 2004; Maruska and Mensinger, 2015). Thus, the question remains how single end organs integrate both vestibular and auditory input. For example, what is the effect of swimming on auditory sensitivity.

It has been postulated that fish possess mechanisms to cancel out self-generated movement to maintain sensitivity to other environment cues. Previous investigations have hypothesized that self-generated movement is filtered by central neurons (Montgomery and Bodznick, 1994; Bell et al., 1997) or that afferent activity is inhibited by efferent fibers (Weeg et al., 2005) allowing fish to remain sensitive to external stimuli. However, consistent efferent modulation has not been observed in the teleost utricle during movement (Highstein and Baker, 1985; Boyle and Highstein, 1990b, 1990a; Boyle et al., 2018b). Additionally, many previous studies have been conducted in immobilized or restrained fishes, which prevented assessment of self-generated movement on afferent activity. Therefore, the question remains whether the utricle retains auditory sensitivity during movement.

The oyster toadfish, *Opsanus tau*, is a well-studied model organism for investigating the inner ear including the semicircular canals (Boyle and Highstein, 1990b; Rabbitt et al., 1995), saccule (Fay and Edds-Walton, 1997a, 1997b) and utricle (Boyle et al., 2001, 2018a, 2018b; Maruska and Mensinger, 2015). Toadfish utricular afferents display an increased sensitivity to translational accelerations following exposure to microgravity during space flight (Boyle et al., 2001), and exhibit high variability in discharge rates and response sensitivities to sinusoidal linear acceleration while affixed to a multi-axis linear and angular acceleration system (Boyle et al., 2018b). The ability to detect conspecific vocalizations is critical for the reproductive success of the toadfish. During late spring, male toadfish establish nests in shallow waters and produce courtship vocalizations, termed a boatwhistle (Fish, 1972). Female toadfish must detect and localize these vocalizations to locate the male (Gray and Winn, 1961; Fish, 1972;

Mensinger et al., 2003) and can detect boatwhistles via the lateral line (Radford and Mensinger, 2014), saccule (Fay and Edds-Walton, 1997b) and utricle (Maruska and Mensinger, 2015); however, the effect of movement on auditory sensitivity remains unknown.

Recording neural activity from free-swimming fish is challenging due to electrode stability, movement artifacts and entanglement with tethers; however, a recently developed implantable micromanipulator has allowed for longer duration chronic recordings in swimming fish (Rogers et al., 2017; Mensinger et al., 2019). The goals of the present study were to record from the toadfish utricular nerve during movement to determine the effect of motion on vestibular and auditory sensitivity.

Materials and Methods

Animal Husbandry

Adult toadfish (N = 12 female, N = 8 male; standard length 29.2 ± 1.8 cm; body mass 759.3 ± 95.2 g; mean ± s.d.) were obtained from the Marine Biological Laboratory in Woods Hole, MA. Fish were kept in large flow-through seawater tanks and maintained at ambient water temperatures (20 ± 2 °C). All experimental procedures conformed to institutional animal care protocols.

Micromanipulator and microwire electrode fabrication

Microwire electrodes were custom fabricated and integrated into a 3D printed implantable micromanipulator (Rogers et al., 2017). The micromanipulator (10 x 10 x 15 mm; 4.4 g) consisted of five parts (base, body, nut, screw drive and electrode holder) that were fabricated with a Formlabs Form 2 3D printer using clear photopolymer resin (Somerville, MA). The electrodes were made by threading three insulated (250 μm outer

dia.), silver-coated multi strand (seven wires per strand, ~25 μm dia.) copper wires (New England Wire Technologies, Lisbon, NH) through the body and electrode holder of the micromanipulator. The wire protruding from the back of the manipulator was soldered to insulated silver wires (320 μm) that terminated into an underwater connecter and sealed with liquid electrical tape (Star brite, Fort Lauderdale, FL). Polyimide tubing (2 mm, 300 μm inner dia.) filled with conductive silver paint (GC Electronics, Rockford, IL) was used to join the each multistrand wire to a 1 cm length of 10% platinum/iridium micro wire (20 μm dia., Sigmund Cohn, Mt. Vernon, NY). The three microwires were placed in a 3 mm segment of polyimide tubing (120 μm inner dia.) to maintain the electrode tips in close proximity with approximately 2 mm of microwire protruding from the tubing. UV light cured glue (Bondic; Aurora, Ontario, Canada) was used to insulate the final assembly and secure the polyimide tubing to the electrode holder. Impedance of all microwire electrodes were determined using an impedance-test unit (FHC, Inc.; Bowdoinham, ME) and only electrodes with an impedance between 0.7 and 1.8 MΩ were used in recordings.

Microwire electrode implant and recording

Toadfish were anaesthetized by immersion in 0.005% tricaine solution and then immobilized with an intramuscular injection of 0.01% pancuronium bromide (600 μg kg^{-1}). Fish were placed within a custom stereotactic aquarium on a vibration isolation table and a small medial incision through the dorsal musculature was made to expose the posterior dorsal surface of the skull. A small craniotomy was made lateral to the sagittal crest to expose the utricular nerve and otolith. The micromanipulator was secured with cyanoacrylate gel to the dorsal surface of the skull. Microwires were implanted into the

utricular nerve anterior to its projection from the anterior ramus of the VIII nerve by manually advancing the screw drive of the micromanipulator. Following electrode implant, the vibration isolation table was moved horizontally to stimulate utricular afferents and confirm the electrode placement. The craniotomy was then sealed with cyanoacrylate gel and the muscle, fascia and epidermis were sutured tightly around the micromanipulator to create a water tight seal. Toadfish were then placed in the experimental tank and allowed to recover for 90 min.

Experimental set-up

The experimental arena consisted of a circular fiberglass tank (350 L; 90 cm dia. × 55 cm; 50 cm water depth) placed on a 5 cm rubber mat on cinderblocks (40 × 20 × 10 cm) to minimize vibrations. An acrylic track (80 x 25 x 1.5 cm) with two parallel rails (80 x 4 x 1.5 cm) positioned 8 cm apart was elevated off the bottom of the arena by two acrylic supports (1.5 cm x 30 cm x 8 cm). The track was subdivided into three 25 cm segments [initial (0-25 cm), middle (25-50 cm), end (50-75 cm)] for analysis. An underwater speaker (Clark Synthesis AQ-339, Littleton, CO) was positioned upright on the bottom of the tank approximately 30 cm perpendicular from the midpoint of the sled track (Figure 1). A USB camera (120 fps; 640 x 480 resolution; Svpro, New York, NY) was positioned 175 cm above the water surface to record fish movements.

Toadfish were allowed to spontaneously swim or were propelled forward on a 3D printed sled (12.5 x 7.5 x 2.5 cm, weight: 125 g). The sled was placed on the underwater track, and fish were affixed to the sled with plastic electrical ties around their mid-section. A custom fabricated R/C motorized (Uxcell, Hong Kong) winch system, with monofilament as the cable, was secured to the upper rim of the tank opposite the fish's

initial position and was used to pull the fish/sled forward at three speeds (slow, medium and fast). The monofilament was threaded through an underwater pulley positioned at track level to insure the sled maintained contact with the track during movement (Figure 1). A minimum of 10 trials/speed were conducted for all toadfish tested.

Sound stimulus

The underwater speaker was connected to a mixer amplifier (Bosch Plena; Farmington Hills, MI), and the sound stimulus consisted of either a continuous 60 s pure tone (125, 150, 175 and 200 Hz) created with a function generator (Model: AFG1022; Tektronix Co. Ltd., Shanghai, China) or the playback of a field recorded toadfish boatwhistle (180 Hz fundamental frequency; 425 ms duration) that was presented at a frequency of 0.25 Hz. Prior to each trial, sound pressure level (dB re. 1 μPa) for all sound stimuli were measured at the midpoint of each track segment using a calibrated hydrophone (HTI-96-MIN, open circuit voltage (OCV) with preamp battery = -165 dB re. 1 V/μPa; High Tech Inc., Long Beach, MS) that was connected to a PowerLab data acquisition system (Model: 8/35; ADInstruments Inc., Colorado Springs, CO). All sound stimuli were presented at a sound pressure level of approximately 130 dB re. 1 μPa. The average root mean square (rms) voltage (V_{rms}) of the sound at the midpoint of each track segment was calculated using a custom Matlab software (Version 2017a) script.

Experimental protocol

Prior to experimental trials, the underwater connector was coupled to a waterproof tether (~3 m) that connected to a differential amplifier (×1000; Dagan, USA). The neural signal was filtered (0.03 to 5 kHz), recorded using Spike2 software (Version 8; Cambridge Electronic Design Ltd, Cambridge, England) and monitored on a portable

computer. Two of the three microwire electrodes from each implant were chosen for recording based on signal fidelity.

During stationary experiments, toadfish were positioned in the center of the tank with the front of the underwater speaker positioned lateral to the center of the toadfish head approximately 30 cm from the ipsilateral side of the implant. Fish were exposed to 60 s of continuous pure tone playbacks ranging from 125 to 200 Hz or 10 boatwhistle playbacks (425 ms duration, fundamental frequency 180 Hz) at a frequency of 0.25 Hz.

For all sled trials, fish were positioned on the far right of the tank (point A) and pulled across the tank (~ 75 cm) to the opposite side (point B) (Figure 1). Alternatively, fish were allowed to swim freely throughout the experimental arena after the track was removed. Utricular activity was recorded with and without sound while toadfish were pulled forward on the sled at slow sled speeds or during free-swimming. For conspecific playbacks, 5 boatwhistles were presented pre- and post-movement, however during transit, there was only sufficient time for one boatwhistle presentation per track segment The angle of fish head in relation to the speaker was approximately at 130° (pre-movement), 130° - 163° (initial), 163° - 196° (middle), 196° - 229° (end) and 229° (post-movement) during playbacks.

Utricular afferent spontaneous firing rates (spikes s^{-1}) were determined for each unit and were calculated as the number of discharges over a given time in the absence of external stimuli or movement. Firing patterns were determined based on the shape of the interspike interval histogram and fibers were characterized as regular (normally distributed interspike histogram) or irregular (non-normal distributed interspike histogram) (Weeg and Bass, 2002). Fiber responses to movement were classified as either

phasic-tonic, which were characterized by an initial increase in firing followed by a sustained response above spontaneous rates throughout movement, or phasic, which were characterized by peak firing rates within one second of movement followed by a return to ± 5% of baseline rates during the remainder of movement.

Neural activity, sound stimulus and overhead videos were recorded using a CED Micro 1401 data acquisition unit, and Spike2 software (Version 8; Cambridge Electronic Design Ltd., Cambridge, England). Individual units were discriminated offline using Spike2 waveform analysis. Toadfish position, linear velocities (v) and linear accelerations (a) were determined using the overhead video with a custom Matlab software (Version 2017a) script using the following equations:

Eq. 1: $v = (x_{i+1} - x_i)/(t_{i+1} - t_i)$,

Eq. 2: $a = (v_{i+1} - v_i)/(t_{i+1} - t_i)$.

Where linear velocity (v) is equal to the change in fish position (x_{i+1} - x_i) over a given time period (t_{i+1} - t_i), while linear acceleration (a) is equal to the change in velocity (v_{i+1} - v_i) over a given time period (t_{i+1} - t_i).

Particle acceleration

Particle acceleration (dB re. 1 ms^{-2}) for sled movements (slow, medium and fast) and boatwhistle playbacks were determined using a calibrated waterproofed triaxial accelerometer (Model: W356A12/NC; Sensitivity: X = 10.47 mV/ms^{-2}; Y = 10.35 mV/ms^{-2}; Z = 10.29 mV/ms^{-2}; PCB Piezotronics, Depew, NY). The triaxial accelerometer was connected to a signal conditioner (Model: 482C; PCB Piezotronics, Depew, NY) and monitored with a PowerLab data acquisition system. To measure particle acceleration during sled movement, the accelerometer was attached to the sled at the position of the

fish's head and pulled along the track at the three sled speeds (slow, medium and fast). Ten trials were conducted and averaged for each speed. To determine particle acceleration levels during boatwhistle playbacks, the accelerometer was made neutrally buoyant using polystyrene insulation sheathing (Zeddies et al., 2012; Cardinal et al., 2018) and suspended at the position of the fish before movement (pre-movement), at the midpoint of each track segment (initial, middle and end) and after movement (post-movement). All measurements (N=10/position) were made approximately 6 cm above the track to correspond with the utricle location. All data was analyzed offline using LabChart software (Version 8; ADInstruments, Colorado Springs, CO). Particle acceleration (dB re. 1 ms^{-2}) was calculated with a custom Matlab software (Version 2017a) script, where the root mean square (rms) voltage (V_{rms}) values of each axis (*X*, *Y* and *Z*) were calibrated to the sensitivity of the accelerometer and used to calculate the magnitude of particle acceleration in the dB scale using the follow equation:

Eq. 3: $dB\ re.1\ ms^{-2} = 20\ Log_{10}(\sqrt{X^2 + Y^2 + Z^2})$

Statistical analysis

Statistical analysis for all utricular afferent firing rates was performed in Matlab (Version 2017a). All data passed the Shapiro-Wilks normality test except particle acceleration, which was analyzed with non-parametric tests. The effect of sled speed (slow, medium and fast) on spontaneous utricular firing (spikes s^{-1}) without sound was determined by conducting a one-way analysis of variance (ANOVA) followed by a Tukey's honestly significant differences (HSD) test to determine if firing rates significantly increased above spontaneous rates at each sled speed.

During sound stimulus, a Student's *t*-test compared evoked (pure tone and boatwhistle playback) and spontaneous utricular firing rates (spikes s^{-1}). The degree of phase locking to the sound stimulus was determined by calculating the coefficient of synchronization (R), where strong phase locking is indicated as $R > 0.50$ and weak phase-locking is represented by $R \leq 0.5$ (Goldberg and Brown, 1969). However, given that a small sample size (N) may misrepresent R, the Raleigh statistic (Z), where Z is a combined measure of the number of discharges (N) and strength of phase locking (R) and is defined as $N \times R^2$, was calculated to determine whether phase locking was statistically significant ($Z > 6.91$; $p < 0.001$) (Lu and Fay, 1993, 1995).

To determine significant differences between sled speed (slow, medium and fast) particle acceleration levels (dB re. 1 ms^{-2}) when sound stimulus was absent, a Kruskal-Wallis one-way ANOVA was conducted followed by a Dunn-Sidak post-hoc test. A Wilcoxon rank-sum test was performed to determine differences in particle acceleration levels during sled movement and boatwhistle playbacks.

Results

Toadfish utricular recordings

Utricular activity from 36 afferent fibers were successfully recorded from 20 toadfish. All units (N = 36) exhibited spontaneous firing rates ranging from 5 to 82 spikes s^{-1} (42 ± 23 spikes s^{-1}; mean ± 1 s.d.; Figure 2A). The firing pattern was comprised predominately of irregular-type fibers (31 units, 86.1%; Figure 2B) with the remainder of the units displaying regular firing (5 units, 13.9%; Figure 2C).

Utricular response to sound (stationary fish)

The firing rate (spikes s^{-1}) of all utricular afferents in stationary toadfish significantly increased (Student's *t*-test, $p < 0.05$) above spontaneous rates for all pure tones and boatwhistles playbacks. Figure 3 shows a representative utricular fiber (TF5-A) that significantly increased (Students *t*-test, $p < 0.001$) firing rates (spikes s^{-1}) (Figure 3A-D), and strongly and significantly phase-locked to 150, 175 and 200 Hz pure tones (150 Hz: R = 0.76, Z = 1387.3; 175 Hz: R = 0.75, Z = 1074.8; 200 Hz: R = 0.61, Z = 1061.1, Figure 3F-H) and weakly phase-locked to 125 Hz (R = 0.29, Z = 241.5, Figure 3E). Figure 3I displays the phase-locking responses of three additional utricular afferents (TF5-B, TF7-A and TF10-A). Additionally, figure 4 shows that utricular afferents significantly increased (Student's *t*-test, $p < 0.05$) their firing rates (spikes s^{-1}) in response to boatwhistle playbacks, and exhibited strong (R = 0.68) and significant phase-locking (Z = 274.8) to the tonal portion of the boatwhistle.

Utricular response to movement (sled)

Duration (s), velocity (cm s^{-1}) and acceleration (cm s^{-2}) of sled movements were inversely correlated to toadfish weight (range 504.9 – 941.4 g), with heavier fish taking slightly longer to travel along the track and thus having lower velocity and acceleration values than lighter fish. At slow speed, movement duration ranged from 13.27 – 14.95 s (14.10 ± 0.53 s), velocity from 3.97 – 5.37 cm s^{-1} (4.97 ± 0.06 cm s^{-1}) and acceleration from 0.32 – 0.41 cm s^{-2} (0.35 ± 0.01 cm s^{-2}); at medium speed, duration ranged from 8.05 – 9.56 s (8.85 ± 0.51 s), velocity from 5.56 – 9.03 cm s^{-1} (8.32 ± 0.53 cm s^{-1}) and acceleration from 0.66 – 1.11 cm s^{-2} (0.95 ± 0.12 cm s^{-2}); at fast speed, duration ranged from 6.04 – 7.33 s (6.70 ± 0.44 s), velocity from 10.20 – 12.23 cm s^{-1} (11.10 ± 0.75 cm s^{-1}) and acceleration from 1.36 – 2.02 cm s^{-2} (1.67 ± 0.22 cm s^{-2}).

All afferents tested during the movement trials (N = 26) increased their firing rates at the onset of movement; however, the sustained response was variable. During sled movement, 76.9% of fibers (N = 20) exhibited a phasic-tonic response, where velocity-sensitive afferents showed a modest decrease in firing after the initial peak but sustained rates above baseline throughout movement. In contrast, acceleration-sensitive afferents (N = 6) exhibited phasic responses that quickly increased their firing rates in phase with acceleration then returned to ±5% of baseline rates throughout the duration of movement.

Figure 5A displays the mean spontaneous and sled evoked neuronal activity (spikes s^{-1}) of five individual afferents, with medium and fast speeds resulting in significant increases in firing rates (Tukey's HSD, $p < 0.05$). Figure 5B and 5C plot the mean firing rates (spikes s^{-1}) of utricle afferents (N = 5) versus velocity (cm s^{-1}) and acceleration (cm s^{-2}), respectively.

Figure 6 illustrates the activity (spikes s^{-1}) of two toadfish utricular afferents (TF27-A & TF29-B) during sled movement. Fiber TF27-A displayed a phasic response pattern, which was correlated with acceleration increase, with evoked rates increasing above baseline rates and then returning to within ± 5% of baseline activity as acceleration decreased (0.92 – 2.58 s). At all three speeds, acceleration peaked within one second of movement onset (slow 0.47 ± 0.08 s; medium 0.70 ± 0.05 s, fast 0.76 ± 0.03 s), with peak accelerations of 6.2 cm s^{-2} (slow), 15.5 cm s^{-2} (medium) and 16.4 cm s^{-2} (fast). Fiber TF29-B displayed phasic-tonic response patterns, with a sharp increase in firing rates during initial acceleration followed by reduced rates throughout movements that remained above baseline. During sled movement, acceleration peaked at 3.9 cm s^{-2} in

0.84 ± 0.12 s (slow), 8.9 cm s^{-2} in 0.54 ± 0.07 s (medium) and 14.9 cm s^{-2} in 0.65 ± 0.04 s (fast). All rates significantly increased (Student's *t*-test, $p < 0.001$) above the spontaneous rates of 27.0 ± 1.8 spikes s^{-1} as follows: 37.1 ± 0.7 spikes s^{-1} (slow), 45.2 ± 1.3 spikes s^{-1} (medium) and 45.0 ± 1.4 spikes s^{-1} (fast). Throughout the duration of movement, firing rates remained elevated above spontaneous rates. During deceleration, firing rates sharply decreased before returning to spontaneous rates while stationary.

Utricular response to sound (sled movement)

All utricular fibers (N = 26), responded to sound during movement. Figure 7 illustrates utricular afferent activity of a phasic-tonic (TF18-B) and phasic fiber (TF26-A) before, during and after movement in the presence or absence of toadfish boatwhistle playbacks. Both afferents exhibited increased spike rates in response to movement and during boatwhistle playbacks. Additionally, all fibers displayed strong ($R > 0.50$) and significant ($Z > 6.91$; $p < 0.001$) phase-locking to the tonal portion of boatwhistle playbacks during movement (N = 10 trials; Figure 8).

Particle acceleration

Figure 9 shows the median particle acceleration levels generated from background levels, during boatwhistle playback and by the three sled speeds during movement. Background median particle acceleration levels were -48.27 dB re. 1 ms^{-2}. Slow (median = -21.89 dB re. 1 ms^{-2}) and medium (median = -18.68 dB re. 1 ms^{-2}) speed particle acceleration levels were significantly lower (Dunn-Sidak, $p < 0.001$;) than during fast speeds (median = -14.09 dB re. 1 ms^{-2}) (Figure 9A). Additionally, boatwhistle particle acceleration levels (median = -15.47 dB re. 1 ms^{-2}) were significantly greater (Wilcoxon rank-sum test, $p < 0.001$) than slow speed levels (Figure 9B).

Free-swimming

The utricular activity (N = 3) of swimming toadfish was determined in the presence and absence of boatwhistle playbacks. Fish exhibited spontaneous swimming that lasted 2 – 15 s and covered distances of 15 to 120 cm, with average linear accelerations ranging from 0.8 to 24.5 cm s^{-2}. For utricular fibers TF17-A and TF27-A, spontaneous rates (TF17-A: 29.2 ± 1.9 spikes s^{-1}; TF27-A: 37.8 ± 3.1 spikes s^{-1}) significantly increased (Student's *t*-test, $p < 0.05$) during swimming to 84.2 ± 15.6 spikes s^{-1} and 54.9 ± 4.5 spikes s^{-1}, respectively. However, for fiber TF32-A, spontaneous rates (43.4 ± 2.1 spikes s^{-1}) did not increase until linear accelerations were greater than 4.5 cm s^{-2} (Figure 10).

Figure 11 shows the neural activity of two utricular afferent fibers (TF17-B and TF32-A) in response to boatwhistle playbacks while stationary and swimming. Two brief swims are monitored for TF17 (Top, Figure 11), with peak velocities of 6.1 and 6.3 cm s^{-1}, respectively, and accelerations 11.3 and 16.8 cm s^{-2}, respectively. Firing rates of TF17-B (44.4 ± 1.4 spikes s^{-1}) significantly increased (Student's *t*-test, $p < 0.001$) to 85.1 ± 2.0 spikes s^{-1} during boatwhistle playbacks while the fish was stationary. During movement, rates increased to 57.9 ± 2.5 spikes s^{-1}, and spiked again in response to boatwhistle playbacks to 115.7 ± 3.9 spikes s^{-1} (Figure 11, Top). Similarly, the firing rates of fiber TF32-A significantly increased (Student's *t*-test, $p < 0.001$) above baseline (25.6 ± 1.6 spikes s^{-1}) to 53.9 ± 0.8 spikes s^{-1} in response to boatwhistle playbacks when stationary, while during swimming rates increased to 37.6 ±2.1 spikes s^{-1} without sound and to 60.5 ± 2.9 spikes s^{-1} during boatwhistle playbacks (Figure 11, Bottom).

Discussion

Utricular afferents responded to both natural swimming and artificial movements, while also retaining sensitivity to detect external auditory stimuli. This study is the first to investigate multimodal encoding of the utricle during self-generated movements from freely-swimming fish.

Unlike terrestrial vertebrates, which have evolved separation of vestibular and auditory organs, fish otoliths respond to both linear movement and sound (Fritzsch, 1999). Therefore, it has remained unclear how fish integrate multimodal input within the otolithic organs such as when a swimming fish encounters external auditory stimuli. It has been postulated that adaptive filters within higher order brain centers can minimize or cancel self-generated movement (Montgomery and Bodznick, 1994; Bell et al., 1997), or that efferent neurons could inhibit afferent neuronal activity (Roberts and Meredith, 1989). For example, in the lateral line, efferent modulation has been noted during gilling (Montgomery and Bodznick, 1994), tail movements (Roberts and Russell, 1972), visual stimuli (Tricas and Highstein, 1991) and fictive sound production (Weeg et al., 2005). In the inner ear, efferent modulation has been observed in the saccule (Weeg et al., 2005) as well as in the semicircular canals during sinusoidal mechanical indentation (Rabbitt et al., 1994) and electrical stimulation (Boyle and Highstein, 1990a). However, these previous studies have been conducted on restrained or stationary fish receiving a single stimulus. Recent studies in the anterior lateral line of toadfish showed no evidence of efferent modulation during swimming (Mensinger et al., 2019). Similarly, utricular efferents were not modulated by sound or gilling (Maruska and Mensinger, 2015), or sinusoidal linear acceleration (Boyle et al., 2018b).

Toadfish are primarily benthic ambush predators and spend the majority of their time under hard substrate. *In-situ* observations of toadfish swimming are complicated by limited visibility in their estuarine habitats; however, captive fish exhibit short distance swimming bouts (~ 2 m) interspersed by stationary periods or rapid predatory strikes of 1 to 2 body lengths at speeds ranging from 3.5 to 18.6 cm s^{-1} (Palmer et al., 2003; Mensinger et al., 2019). Faster speeds, which may be expected during startle or escape responses, are rarely observed as threatened toadfish often flare their operculum and retreat into their habitat rather than swim away. The variability in swimming motivation, speed and direction made it difficult to have fish approach the sound source consistently at the same angle and speed. Therefore, toadfish were moved via a sled to allow for a precise correlation of utricular activity with speed and distance from the speaker. The sled velocities (4.0 – 12.2 cm s^{-1}) were within the range of toadfish swimming speeds.

All toadfish utricular afferents rapidly (< 1 s) increased their firing rates in response to movements. Utricular afferents exhibited two distinct response patterns: phasic and phasic-tonic, which were directly correlated with acceleration and velocity, respectively. Phasic responses have been observed previously in the toadfish inner ear during mechanical indentation of the vestibular labyrinth (Rabbitt et al., 1994, 1995, 1999) and during sinusoidal linear acceleration while toadfish were affixed to a multi-axis linear and angular acceleration system (Boyle et al., 2018b). In contrast, phasic-tonic response patterns have not been described in the toadfish, which was likely due to the low frequency (1 – 3 Hz) stimulus presentation in these previous studies. However, phasic-tonic discharge responses have been noted in utricular afferent nerves of the thornback ray (*Raja clavate*) (Lowenstein and Roberts, 1949) and guitar fish (*Rhinobates*

productus) (Macadar et al., 1975, 1978; Macadar and Budelli, 1984) in response to sustained sinusoidal tilts. Similarly, phasic-tonic discharge patterns, without evidence of efferent modulation, have been observed while recording from vestibular nuclei in head-restrained rhesus monkeys (*Macaca mulatta*) during turntable movements (Roy and Cullen, 2001). In both *Xenopus laevis* and *M. mulatta*, it was noted that efferent modulation does not occur without sensory motor command input (Roy and Cullen, 2001; Chagnaud et al., 2015), thus it was crucial to determine utricular activity during self-generated movements.

The utricle is extremely sensitive to movements; as gill movements in stationary fish that show no other external movement, produce robust responses (Maruska and Mensinger, 2015). Swimming did not appear to saturate utricular afferents as sound stimuli increased the firing rate above the levels evoked by movement. The quickly adapting phasic fibers were well suited to detect sound as they returned rapidly to baseline firing levels and remained sensitive to sound input, while the decrease in sensitivity to movement for the phasic-tonic fibers after initial movement also allowed afferents to react to subsequent sound stimulus. While the methodology could not determine the exact mechanisms that led to decreased firing rates, it is possible that the stereocilia of the hair cells were not maximally displaced and/or quickly reset after initial movement (Collin et al., 2000; McHenry and Netten, 2007). Alternatively, efferent modulation may have immediately reduced the sensitivity of phasic fibers and partially decreased the sensitivity of phasic-tonic fibers. However, similar results were observed in both free-swimming and sled fish, and if efferent modulation was occurring, it would not have been expected to be observed in the sled fish as they were not receiving motor

neuron activation during swimming. This would be consistent with lateral line recordings from free-swimming toadfish, which showed no evidence of efferent modulation (Mensinger et al., 2019).

While this study allowed for the assessment of utricular auditory and vestibular sensitivity during movement, several limitations of this study must be addressed. For example, the size of the tank (90 cm dia.) limited the distance of movement and complicated the integrity of the acoustical stimulus by causing echoes and reverberations that may have occurred at the tank edges (Rogers et al., 2016). However, the utricle responded in phase to both pure tones and boatwhistles vocalization playbacks indicating the sound retained most of its integrity, and the afferent neural responses were closely correlated with stimulus durations suggesting that echoes were limited. Future experiments in larger tanks or in the field should be conducted to more accurately address the range of sound detection and how the utricle encodes particle motion gradients as fish approach sound sources.

This study demonstrates that the toadfish utricle is sensitive to multimodal input from movement and sound. Utricular afferents responded quickly to movement input by increasing firing rates, which then returned partially (phasic-tonic) of fully (phasic) to baseline rates. Thus, swimming fish retained the capacity to detect external sound via the utricle immediately after movement onset showing division of vestibular and auditory in a single otolithic end organ.

Acknowledgments: The authors would like to acknowledge the Marine Resources Center staff at the Marine Biological Laboratory for providing toadfish care. Funding was

provided by National Science Foundation Grants IOS 1354745 and DBI 1359230 & 1659604 to AFM, and by the National Science Foundation Graduate Research Fellowship Program under grant DGE 1804377 to LSR.

Figures

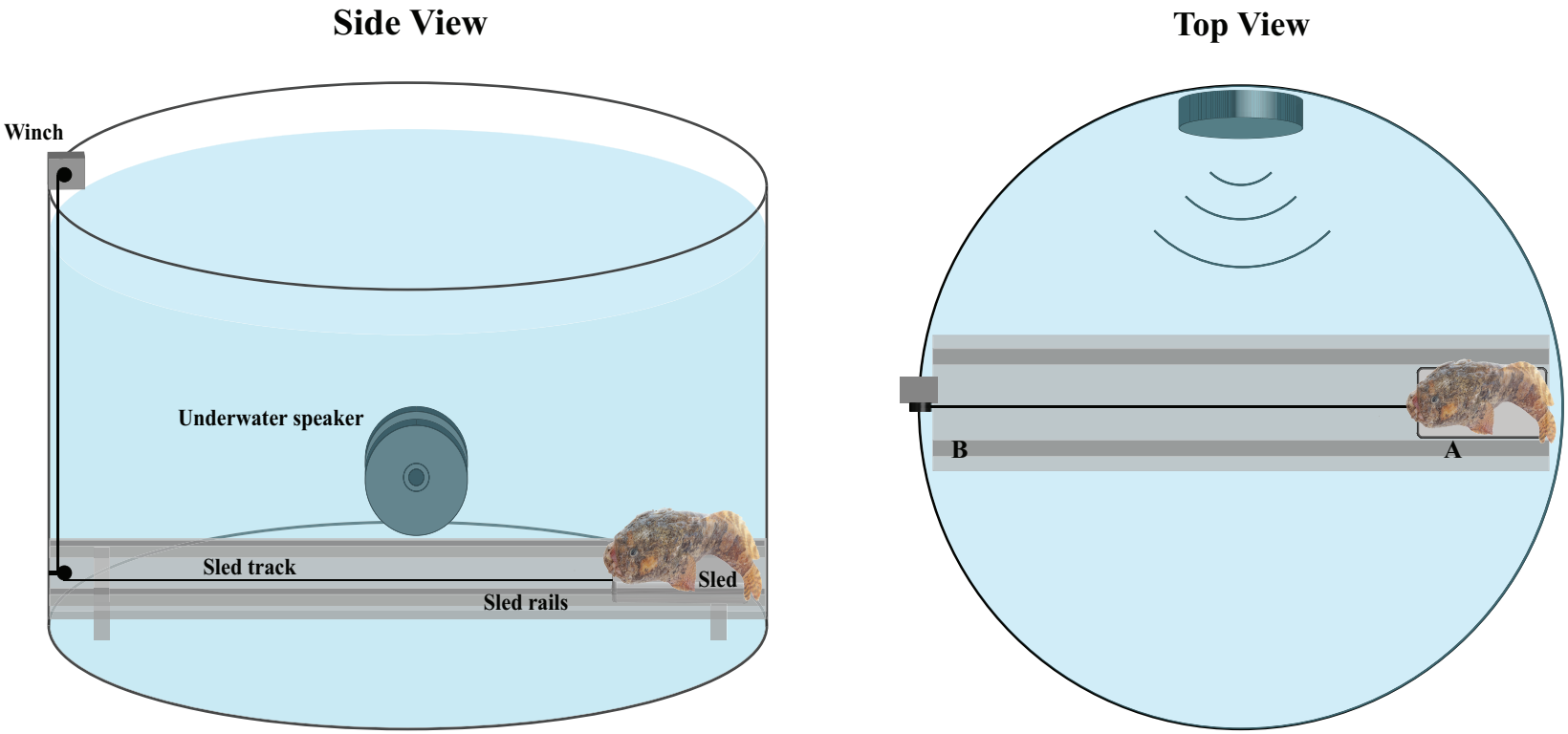

Figure 1: Side (left) and top (right) views of experimental tank. Toadfish were pulled (~ 75 cm) from point A to point B while attached to a 3-D printed sled (12.5 x 7.5 x 2.5 cm) on a track by a motorized winch system at variable speeds (slow, medium and fast). An underwater speaker was positioned vertically approximately 30 cm perpendicular from the midpoint of the sled track. All sound stimuli were presented at a sound pressure level of approximately 130 dB re. 1 µPa.

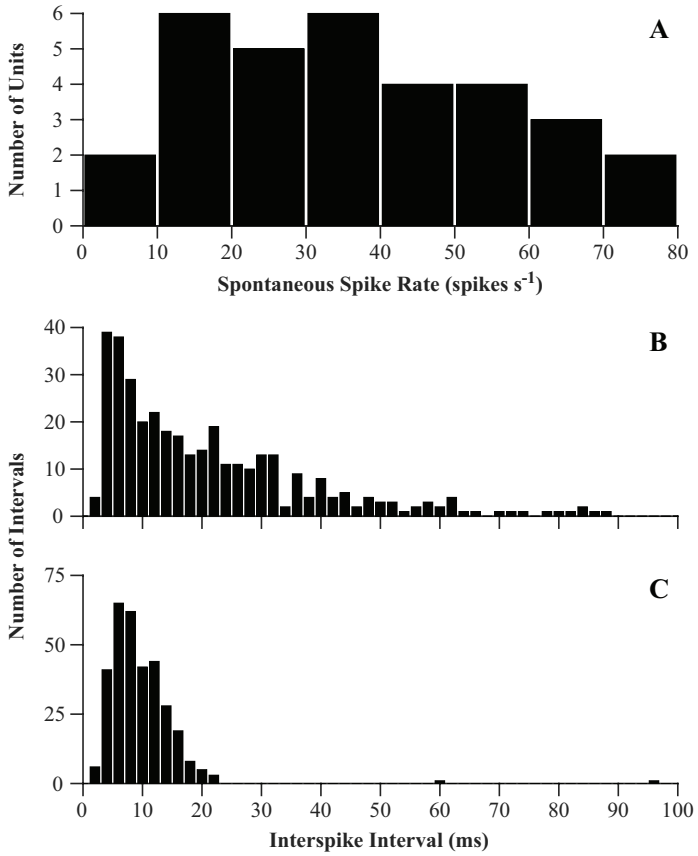

Figure 2: Spontaneous activity of afferent utricular fibers (N=36). (A) Histogram of spontaneous spike rates (spikes s^{-1}) binned in 10 spikes s^{-1} increments. (B) Interspike interval histograms of representative irregular (31 units, 86.1%) and (C) regular (5 units, 13.9%) afferent firing patterns. Interspike interval histograms were generated using a minimum of 300 spikes and were grouped in 2 ms bins.

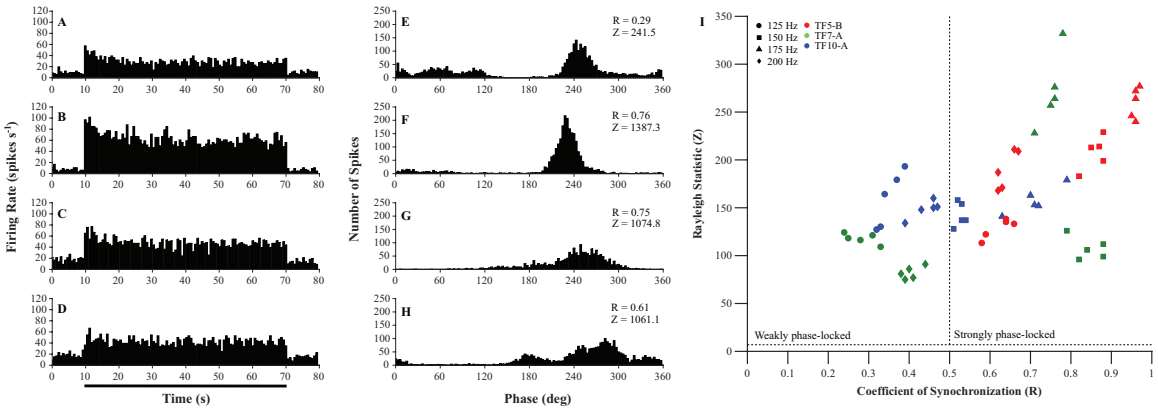

Figure 3: Toadfish utricular afferent response (N = 4 afferents) to pure tone stimuli (130 dB re. 1 μPa). Peri-stimulus time histograms (A – D) plot firing rate versus time and phase histograms (E – H) plot the total number of spikes versus one sinusoidal cycle for a representative utricular afferent in response to pure tone stimulus (black horizontal bar = 60 s duration). 125 Hz (A & E), 150 Hz (B & F), 175 Hz (C & G) and 200 Hz (D & H). Peri-stimulus time histograms are binned in 1 sec increments, while phase histograms are binned in 3 degree increments. (I) Rayleigh statistic (Z) plotted against the coefficient of synchronization (R) for toadfish afferents (N = 3) in response to 125 (o), 150 (□), 175 (Δ) and 200 Hz (◊) pure tone stimulus (60 s duration). Each afferent is plotted with a different color (red, blue or green). The vertical dashed line (R = 0.5) indicates the divide between weak (R < 0.5) and strong (R > 0.5) phase-locking, while the horizontal line (Z = 6.91) represents the divide between significant (Z > 6.91, $p < 0.001$) and non-significant (Z < 6.91, $p > 0.001$) phase-locking.

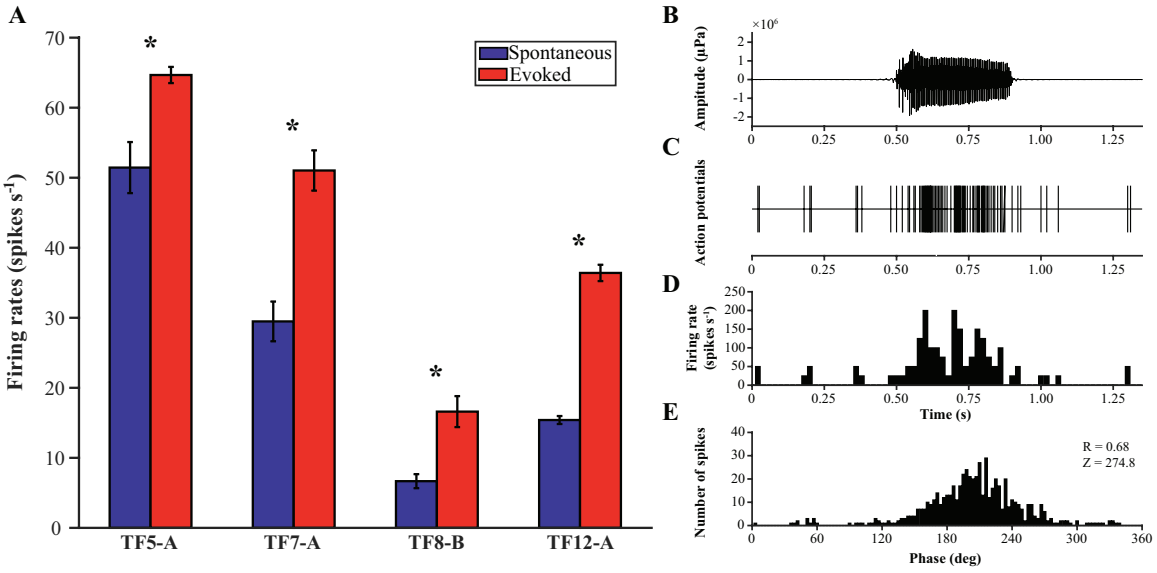

Figure 4: Utricular response of stationary toadfish to boatwhistle playbacks (130 dB re. 1 μPa). (A) Mean spontaneous (blue) and evoked (red) firing rates (spikes s^{-1}) of 4 utricular afferent fibers. Evoked firing rates (spikes s^{-1}) are the average response to 10 boatwhistle playbacks, while spontaneous firing rates are the average utricular activity (2 s) preceding each playback. Error bars represent ± 1 standard deviation. Asterisks indicate significant increase in evoked firing rates compared to spontaneous firing rates (Student's t-test, $p < 0.05$). (B) Waveform of boatwhistle vocalization plotted versus time (s). (C) Neural activity plotted versus time (s), with vertical lines representing action potentials. (D) Peri-stimulus time histogram binned in 2 ms increments. (E) Phase histogram in response to 10 boatwhistle playbacks plotted in 3-degree increment bins.

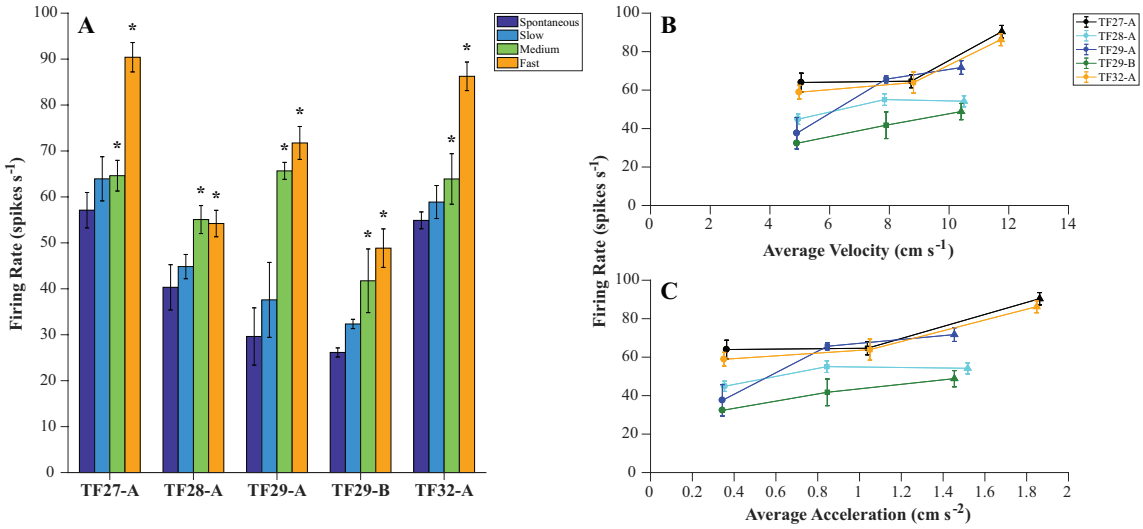

Figure 5: Mean firing rates of utricular afferents (N=5) during sled movements (N=10 trials). (A) Bar graph plots mean firing rate (spikes s^{-1}) for spontaneous (purple), slow (blue), medium (green) and fast (orange) sled movement. Error bars represent ± 1 standard deviation. Asterisks indicate a significant difference in evoked rates from spontaneous rates (Tukey's HSD, $p < 0.001$). (B) Mean linear velocity (cm s^{-1}) and (C) acceleration (cm s^{-2}) plotted against the average toadfish utricular firing rates (spikes s^{-1}) during slow (●), medium (■) and fast (▲) sled movement. Lines connecting individual toadfish spike rates (spikes s^{-1}) are for illustrative purposes only.

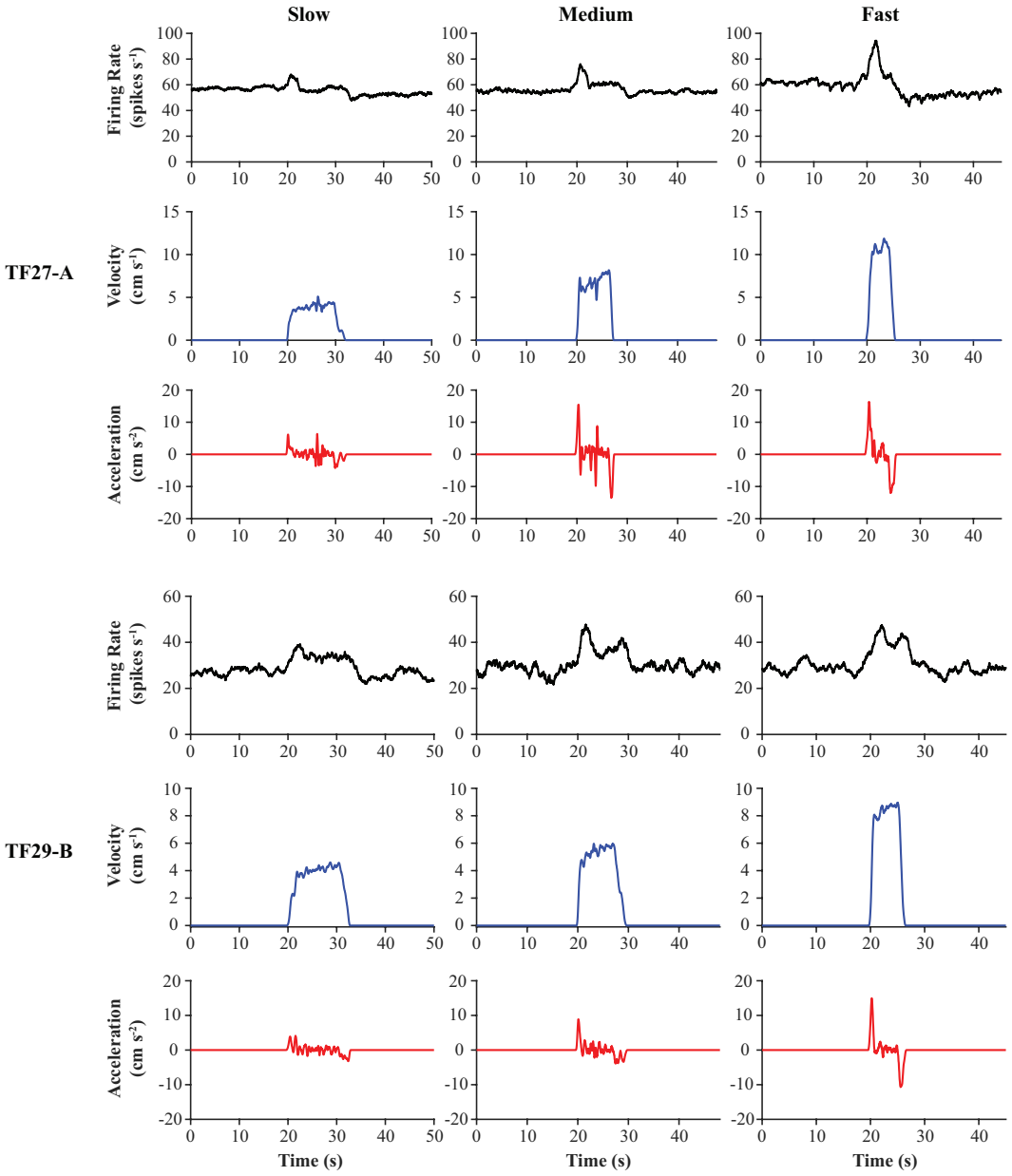

Figure 6: Utricular firing rates (spikes s^{-1}) of two toadfish utricular afferents (TF27-A and TF29-B) before, during and after sled movement at three speeds (slow, medium and fast). Each panel (from top to bottom) represents the utricular firing rates (spikes s^{-1}; black) during sled movement, instantaneous linear velocities (cm s^{-1}; blue) and accelerations (cm s^{-2}; red).

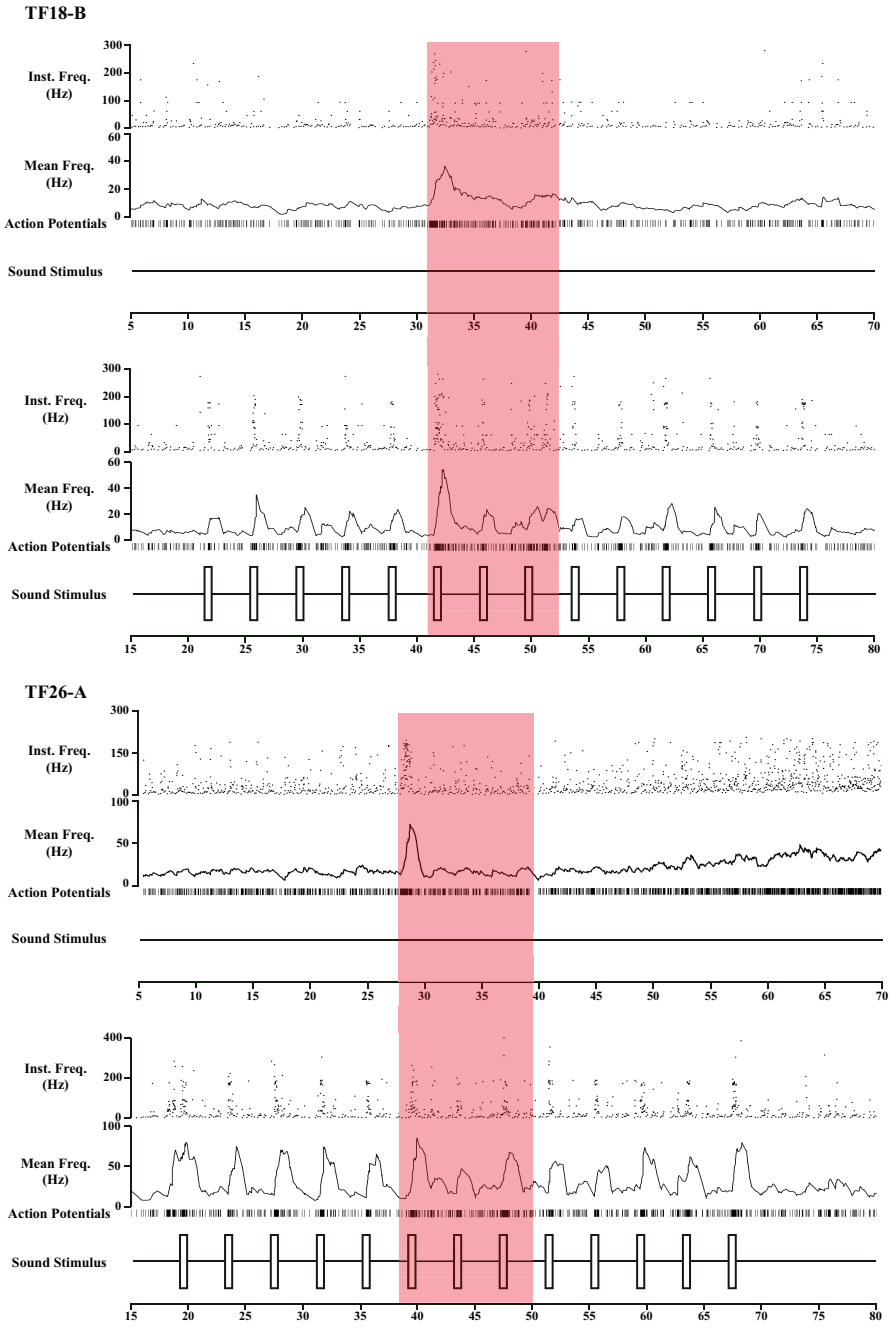

Figure 7: Representative utricular afferent recordings during slow sled movement. Each panel from top to bottom displays instantaneous firing frequency (Hz, spikes s^{-1}), mean firing frequency (Hz, spikes s^{-1}), neural activity (action potentials = vertical lines) and sound stimulus. The shaded red regions indicate when toadfish were moving.

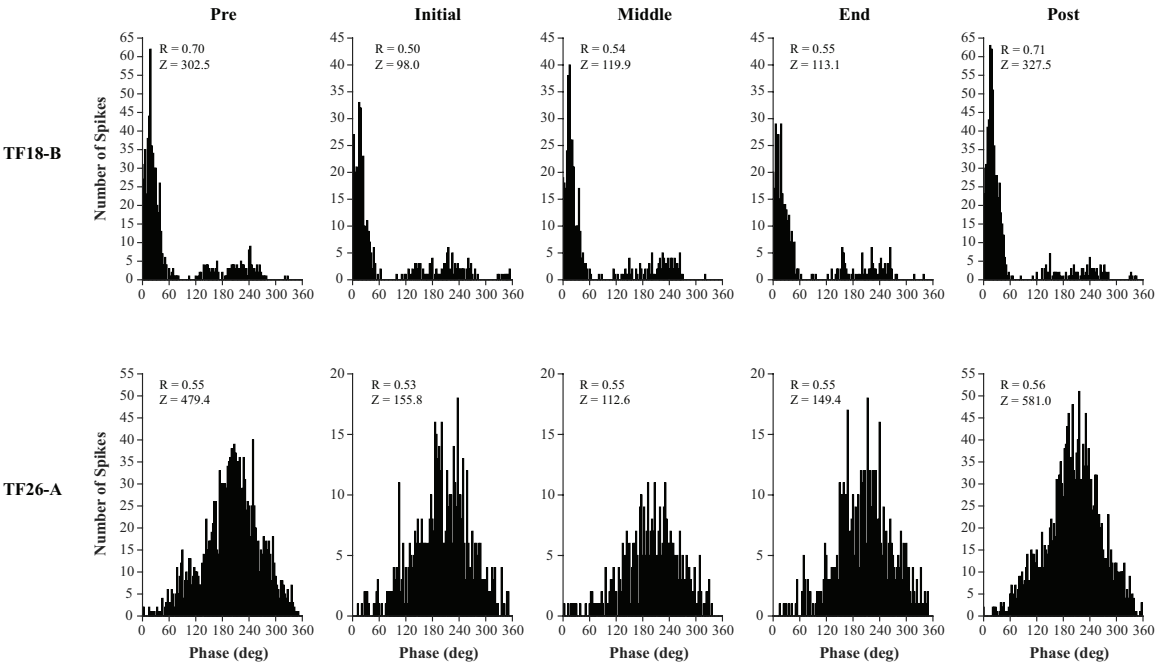

Figure 8: Phase histograms for utricular afferents TF18-B (top) and TF26-A (bottom) in response to boatwhistle playbacks (N =10) during slow sled movement. The position of toadfish within the tank are as follows: Pre-movement (0 cm), initial movement (0 – 25 cm), middle movement (25 to 50 cm), end movement (50 to 75 cm), and post-movement (75 cm). Fish were presented with boatwhistle playbacks at 130° (bearing from speaker, pre-movement), 130° - 163° (initial movement), 163° - 196° (middle movement), 196° - 229° (end movement) and 299° (post-movement). R represents the coefficient of synchronization, where strong phase locking is represented by $R > 0.50$ and Z represents the Raleigh statistic, where $Z > 6.91$ and indicates significant phase-locking ($P < 0.001$). Phase histograms are binned in 3 degree increments. Note the different spike scales.

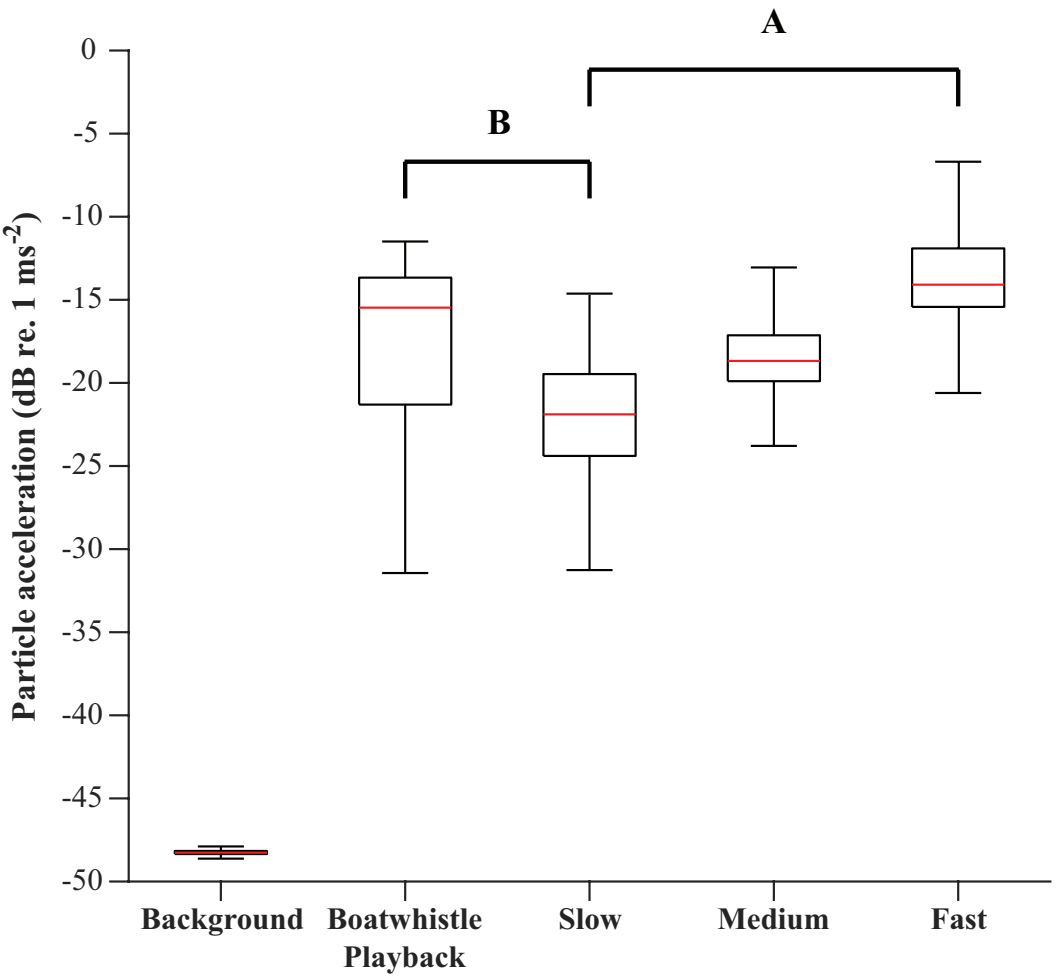

Figure 9: Particle acceleration level (dB re. 1 ms^{-2}) measurements. (A) Boxplots of the particle acceleration levels during slow, medium and fast sled movement, which were used to compare the particle accelerations among the three tested sled speeds (Kruskal-Wallis ANOVA). (B) Boxplots of the particle acceleration levels during slow sled movement and boatwhistle playbacks to compare particle acceleration levels during moving boatwhistle playback experimental trials (Wilcoxon rank-sum test). Background indicates the particle acceleration levels (dB re. 1 ms^{-2}) within the experimental when all stimulus is absent. Red bars represent median, while upper and lower tails signify 95th and 5th percentiles, respectively.

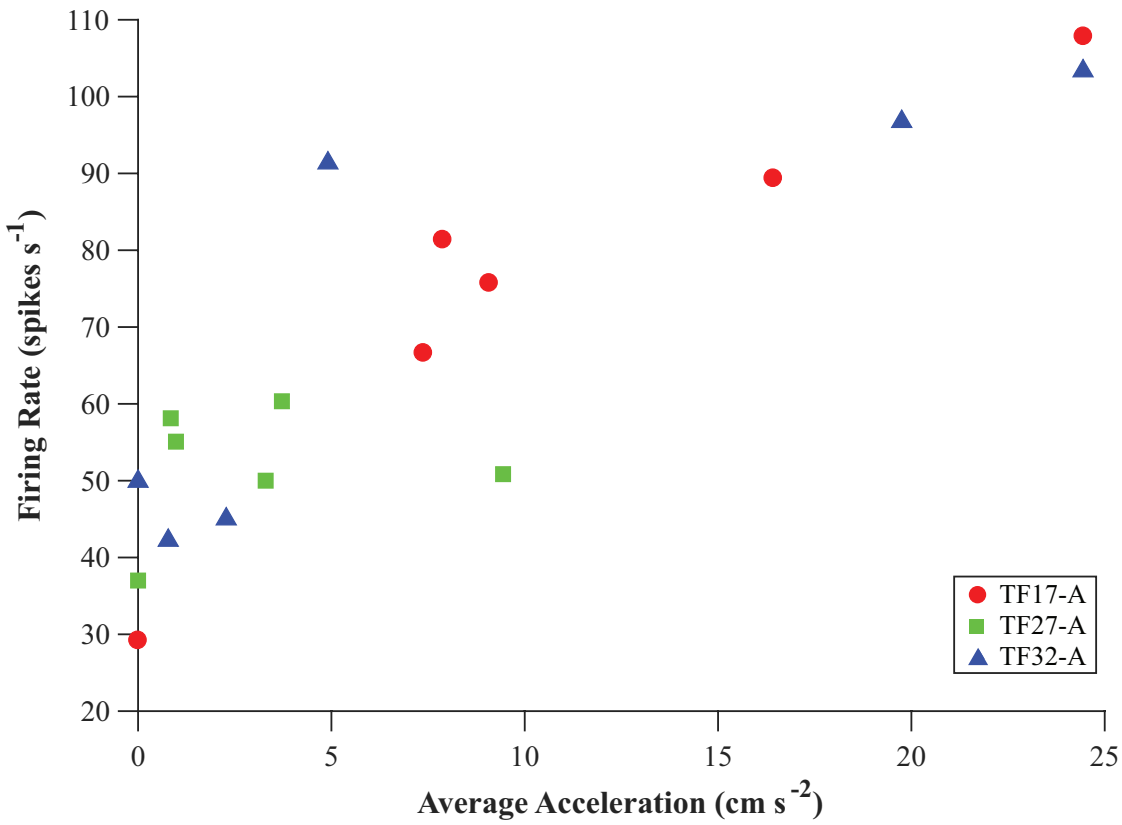

Figure 10: Mean toadfish utricular afferent firing rates (spikes s^{-1}) during forward swimming. Each data point represents an individual movement event.

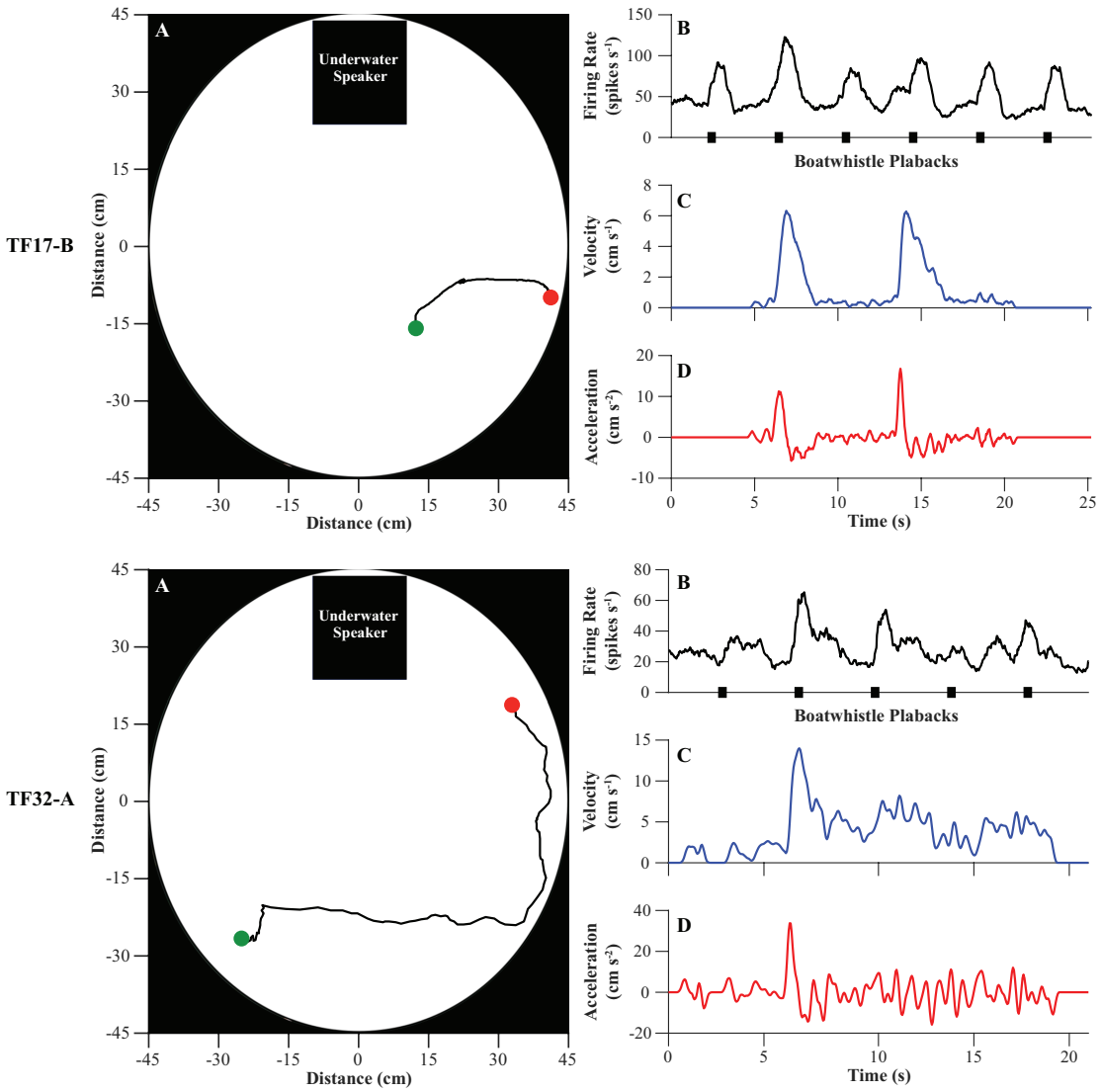

Figure 11: Two free swimming toadfish utricular afferent activities (TF17-B & TF32-A) during boatwhistle playbacks. (A) Experimental arena. Black lines represent path of toadfish swimming during each swimming event from the start of movement (green circle) to the end of movement (red circle). Black box indicates the position of the underwater speaker during each swimming event. (B) Mean firing rate (spikes s^{-1}) during free-swimming in response to a 180 Hz boatwhistle playback. Black boxes on x-axis indicate when boatwhistle playbacks occurred (0.25 Hz). (C) Instantaneous linear velocity (cm s^{-1}; blue) and (D) acceleration (cm s^{-2}; red) for each free-swimming event.

Chapter 4: Future Investigation for Fish Sound Detection

From previous behavioral and physiological investigations, it is evident that both the inner ear and lateral line play a role in the ability of fish to localize sound sources (Buwalda et al., 1983; Sisneros and Bass, 2003; Zeddies et al., 2010, 2012; Radford and Mensinger, 2014; Maruska and Mensinger, 2015; Cardinal et al., 2018; Mensinger et al., 2019). Given that both sensory systems are innervated by hair cells, which display variations in directional sensitivities, it is likely that both the inner ear and lateral line detect particle motion components of sound stimuli. However, the exact mechanisms and the contribution of each sensory system in sound source localizations remains unknown. Therefore, an integrative approach that combines both behavioral and physiological approaches must be utilized to further understand the sound source localizations abilities of fishes.

In the toadfish, both the inner ear and lateral line have been shown to detect vibrational and pure tone stimuli, as well as playbacks of conspecific vocalizations under controlled laboratory settings (Fay and Edds-Walton, 1997a, 1997b; Radford and Mensinger, 2014; Maruska and Mensinger, 2015; Cardinal et al., 2018). For example, Cardinal et al. (2018) utilized bidirectional chronic neural recordings to show that interaural time differences between lateral line neural spikes may play a role in the detection of conspecific vocalizations (Cardinal et al., 2018). Additionally, with advancements in conducting long-term chronic neural recordings, it has recently been shown that the lateral line (Mensinger et al., 2019) is capable of detecting vibration stimuli and that the inner ear (Chapter 3) is sensitive to boatwhistle vocalization

playbacks during self-generated movement. While each of these studies has significantly contributed to the understanding of fish sound source detection, positive phonotaxic behaviors within controlled laboratory studies were not observed.

Unlike the toadfish, plainfin midshipman (*Porichthys notatus*) have shown to exhibit a robust positive phonotactic response within controlled laboratory settings. Plainfin midshipman behavioral studies investigating sound source localization have shown that fish will localize a conspecific sound source by following the direction of particle motion emitted from the sound source (Zeddies et al., 2010, 2012). By utilizing similar behavioral experimental techniques and by removing various inner ear otolith end organs or the ablation of the lateral line significance of each system in behavioral sound source localization could be identified. While conducting these studies will provide significant understanding of the role each organ serves in sound source localization, physiology must still be accounted for.

Previous investigations have primarily used either behavioral or physiological approaches and have significantly contributed to aquatic neuroethologists understanding of how fishes detect or localize sound. However, by taking an integrative approach to this question, utilizing similar chronic neural recording techniques as those used in Chapter 3 and behavioral techniques employed during plainfin midshipman sound source localization experiments, it may simultaneously be determined how fish behaviorally and physiologically localize sound sources. Since it is likely that each of the otolithic end organs and lateral line simultaneously play a role in sound source localization, a physiological technique that allows for both sensory systems to remain intact during such

behaviors and allow for the monitoring of neural activity will provide further insight into fish sound source localization abilities.

Once understanding how fishes localize underwater sound sources in a controlled experimental environment, other sound sources that make up the acoustic soundscape and their impact on sound source localizations abilities may be investigated. For example, in Chapter 2 exposure to high intensity anthropogenic sound (~150 dB re. 1μPa) was determined to cause significant auditory threshold shifts. These shifts in auditory thresholds likely detrimentally impact the ability of fishes to detect and localize sound sources. By conducting similar integrative studies proposed earlier and incorporating playbacks of anthropogenic sound, the influence of anthropogenic masking could be investigated.

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